Role of p65 Acetylation on NF-κB-Dependent Gene Expression

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Karin M. Rothgiesser

aus

Lima, Peru

Promotionskomitee

Prof. Dr. Dr. Michael O. Hottiger

(Vorsitz und Leitung der Dissertation)

Prof. Dr. Bernhard Dichtl

Dr. Andreas Ladurner

Zürich, 2009

TABLE OF CONTENTS

SUMMARY

To allow the correct functioning of an organism and to adapt to physiological and environmental changes, the expression of a specific subset of genes in each different cell type has to be tightly regulated. Nuclear factor κB (NF-κB) is a family of inducible transcription factors able to drive the transcription of hundreds of genes implicated in the regulation of several cellular processes such as innate and acquired immunity, cell proliferation and apoptosis. The aim of this thesis was to investigate how acetylation of p65, the best-studied subunit of NF-κB, affects NF-κB-driven transcription.

Our results indicate that p65 is acetylated by p300 *in vitro* and *in vivo* at lysines 310, 314 and 315. Acetylation of p65 does not influence nuclear-cytoplasmic shuttling, DNA binding or cell viability in response to TNFα. Genome-wide gene expression analysis revealed that p65 acetylation seems to play a role in NF-κB-dependent gene expression. Real-time RT-PCR analysis of specific genes suggest that acetylation at lysine 310 is required for full activation of a subset of genes, while acetylation at lysines 314 and 315 is needed for the decreased expression of another group of genes.

We also identified SIRT2 as a novel p65 deacetylase. SIRT2 belongs to the family of NAD^+-dependent deacetylases called sirtuins. Deacetylation assays performed *in vitro* or in HEK 293T cells with overexpressed proteins revealed that SIRT2 deacetylates p65 at all three lysines 310, 314 and 315. Furthermore, p65 is hyperacetylated at lysine 310 in *Sirt2*$^{-/-}$ MEFs after TNFα stimulation *in vivo*. Both endogenous proteins interact in the cytoplasm of Jurkat T-cells under basal conditions. While p65 shuttles to the nucleus in response to TNFα stimulation, SIRT2 stays in the cytoplasm, implying that SIRT2 deacetylates p65 in the cytoplasm. Furthermore, gene expression of *Mpa2l*, a gene found to require acetylation of p65 at K310 for its proper expression, was misregulated in *Sirt2*$^{-/-}$ MEFs.

In addition, we studied the role of CARM1 as a transcriptional coactivator of NF-κB. A subset of NF-κB-dependent genes that require CARM1, but not its enzymatic activity, for their proper expression was identified.

Together, our data suggests that p65 acetylation regulates the expression of specific NF-κB-dependent genes and that this is in part controlled by SIRT2-mediated p65 deacetylation.

ZUSAMMENFASSUNG

Damit ein Organismus korrekt funktioniert und sich an Umwelteinflüsse und intrazelluläre Veränderungen anpassen kann, muss die Expression spezifischer Gene in jedem Zelltyp fest reguliert sein. Nukleärer Faktor κB (NF-κB) ist eine Familie von induzierbaren Transkriptionsfaktoren, welche die Expression von Hunderten von Genen steuern kann, die in verschiedenen zellulären Prozessen wie der angeborenen und erworbenen Immunität, der Zellvermehrung sowie der Apoptose, beteiligt sind. Das Ziel dieser Doktorarbeit war es zu untersuchen, wie die Acetylierung von p65, einer der wichtigsten Untereinheiten von NF-κB, die Funktion von NF-κB beeinflusst.

Unsere Ergebnisse zeigen, dass p65 von p300 *in vitro* und *in vivo* an drei Lysinen 310, 314 und 315 acetyliert wird. Die Acetylierung von p65 hatte keinen Einfluss auf die Translokation von p65 in den Nukleus, auf die DNA Bindung oder auf das Überleben der Zellen nach Stimulierung mit TNFα. Eine genom-weite Genexpressionsanalyse deutete darauf hin, dass die Acetylierung von p65 eine wichtige Rolle in der Modulierung der NF-κB-abhängigen Genexpression spielt. Real-time RT-PCR Analysen von verschiedenen Genen zeigen, dass die Acetylierung von Lysin 310 für die vollständige Expression einiger Gene erforderlich ist, während die Acetylierung von Lysin 314 und 315 für die reduzierte Expression anderer Gene benötigt wird.

Zusätzlich haben wir SIRT2 als neue p65 Deacetylase identifiziert. SIRT2 gehört zu den Sirtuinen, einer Familie von NAD^+-abhängigen Deacetylasen. Deacetylierungs-experimente *in vitro* oder in Zellen mit überexprimierten Proteinen zeigten, dass SIRT2 p65 an allen drei Lysinen 310, 314 und 315 deacetyliert. Ausserdem war p65 an Lysine 310 in *Sirt2*$^{-/-}$ MEFs nach Stimulierung mit TNFα hyperacetyliert. Endogenes p65 und SIRT2 interagierten im Zytoplasma von unstimulierten Jurkat T-Zellen. Während sich p65 nach Stimulierung mit TNFα in den Nukleus translozierte, blieb SIRT2 im Zytoplasma. Dies deutet darauf hin, dass SIRT2 p65 im Zytoplasma deacetyliert. Die Genexpression von *Mpa2l*, für dessen richtige Expression die Acetylierung von p65 an K310 benötigt wird, war in *Sirt2*$^{-/-}$ MEFs dereguliert.

Zusammengefasst sprechen unsere Ergebnisse dafür, dass die Acetylierung von p65 die Expression von bestimmten NF-κB-abhängigen Genen reguliert und dass dies zum Teil durch SIRT2-vermittelte Deacetylierung von p65 kontrolliert wird.

ABBREVIATIONS

aa	Amino acids
acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAFF	B-cell activating factor
bp	Base pair
Brd4	Bromodomain containing 4
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	cAMP-response element-binding (CREB) binding protein
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CK2	Casein kinase 2
CMV	Cytomegalovirus
CTD	Carboxy-terminal domain of Pol II
C-terminal	Carboxy terminal
DNA	Deoxyribonucleic acid
FoxO	Forkhead box O
GCN5	General control non-derepressible 5
GDH	Glutamate dehydrogenase
GSK3β	Glycogen synthase kinase 3β
GTFs	General transcription factors
H	Histone
HAT	Histone acetyltransferase
Hda1	Histone deacetylase A 1
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HIF-1α	Hypoxia-inducible factor 1, alpha subunit
HIV-1	Human immunodeficiency virus type 1
HP1	Heterochromatin protein 1
IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL-1β	Interleukin-1β
IRF	Interferon regulatory factor
K	Lysine
kB	kilo bases
kDa	kilo Dalton
LPS	Lipopolysaccharides
LTβ	Lymphotoxin-β
MEFs	Mouse embryonic fibroblasts
mRNA	Messenger RNA
MSK1/2	Mitogen- and stress-activated protein kinase

MYBBP1a	Myb binding protein 1a
NAD^+	Nicotinamide adenine dinucleotide
Nam	Nicotinamide
N-CoR	Nuclear receptor corepressor
ncRNA	Non-coding RNA
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NLS	nuclear localization signal
N-terminal	Amino terminal
OAADPr	2'-O-acetyl-ADP ribose
p300	E1A-associated protein of 300 kDa
PARP1	Poly(ADP-ribose) polymerase 1
PCAF	p300/CBP-associated factor
PHD	Plant-Homeo domain
PIC	Pre-initiation complex
PKA	Protein kinase A
Pol II	RNA polymerase II
PRMT	Protein arginine N-methyltransferase
P-TEFb	Positive transcription elongation factor b
rDNA	ribosomal DNA
RHD	Rel Homology Domain
RNA	Ribonucleic acid
Rpd3	Reduced-potassium-dependency gene 3
RSK1	Ribosomal S6 kinase 1
RT-PCR	Reverse transcriptase polymerase chain reaction
SIR	Silent information regulator
SIRT	Sir2-related protein (sirutin)
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptors
SRC-1	Steroid receptor co-activator 1
TAD	Transactivation domain
TAFs	TBP-associated factors
TBP	TATA box binding protein
TFII	Transcription factor of Pol II
Tip60	Tat interacting protein 60 kDa
TNFα	Tumor necrosis alpha
TSA	Trichostatin A
TSS	Transcription start site

INTRODUCTION

1 RNA polymerase II dependent transcription

The execution of biological processes such as development, differentiation and proliferation, requires the precise and orchestrated expression of genes. The genetic information of DNA is transcribed into different forms of RNA in a highly coordinated process known as transcription (reviewed in [1]). The cellular enzymes responsible for this synthesis are RNA polymerases (Pol). Three main RNA polymerases have been identified in higher eukaryotes, including RNA Pol I, II and III [2]. Pol I is primarily implicated in transcribing 18S and 28S ribosomal RNAs, whereas Pol II is responsible for all messenger RNA (mRNA) synthesis in eukaryotes [3], and Pol III is involved in the synthesis of cellular 5S sRNA, tRNAs and adenovirus VA RNAs [4].

In eukaryotes, transcription of protein-coding genes by Pol II proceeds through multiple steps that include the formation of a pre-initiation complex (PIC), initiation, promoter clearance, elongation and termination of transcription [5]. The expression of protein-coding genes is not only regulated at the level of transcription, but also through mRNA processing and transport, translation and mRNA stability (reviewed in [1]). The major regulatory step is thought to occur at the level of transcriptional initiation.

Eukaryotic Pol II dependent transcription usually requires several factors classified into three groups: general transcription factors (GTFs), promoter-specific transcription factors, and cofactors, which will be discussed in the following sections.

1.1 The general transcription machinery

To achieve accurate transcription *in vitro*, a minimum number of proteins is required, including Pol II and general transcription factors (GTFs) (reviewed in [4]).

1.1.1 RNA polymerase II

The polymerase that synthesizes protein-coding messenger RNA (mRNA) in eukaryotes is Pol II. Yeast and human Pol II consists of 12 different subunits, designated RPB1 to RPB12 [6]. In higher eukaryotes, the carboxyl terminal domain (CTD) of RPB1, the largest subunit of Pol II, contains a tandem repeat of the heptapeptide sequence YSPTSPS, with 52 copies in mammals and 26 to 29 in yeasts [7]. Posttranslational modifications in CTD are crucial for transcriptional regulation at multiple steps. For example, CTD phosphorylation at mainly serines 2 and 5 constitutes a major regulation step in transcription: Pol II involved in PIC assembly is unphosphorylated, whereas highly phosphorylated CTD is required for transcript initiation, elongation and termination (reviewed in [5]). More specifically, Pol II phosphorylation at serine 5 associates with promoter-proximal regions of transcribed genes, whereas phosphorylation at serine 2 increases toward the 3'-end of the gene. Several stress-associated and cyclin-dependent kinases (CDK) have been described to phosphorylate CTD: serine 5 is phosphorylated by CDK7 present in TFIIH and by CDK8 found in Mediator, while serine 2 is phosphorylated by CDK9 associated with positive transcription elongation factor b (P-TEFb). A recent study shows that unphosphorylated CTD binds to DNA, and that this complex is recognized by CDK7, which can then phosphorylate CTD at serine 5 [8]. Upon phosphorylation, CTD dissociates from DNA, allowing transcriptional activation. The CTD then becomes accessible to CDK9 to phosphorylate serine 2, resulting in transition to transcription elongation. Phosphorylation at serine 7 has been recently shown to be important for small nuclear RNAs (snRNAs) maturation [9].

Unlike prokaryotic enzymes, eukaryotic Pol II is not able to recognize the promoters by itself and to drive transcription *in vitro* [10]. For site-specific initiation, additional proteins, called the general transcription factors (GTFs), are necessary.

1.1.2 General transcription factors

Recruitment of Pol II to the transcription start site (TSS) is facilitated by GTFs, known as TFIIA, -B, -D, -E, -F and –H, which assemble at the core promoter in a stepwise manner to form a pre-initiation complex (PIC) [10, 11]. Early *in vitro* experiments determined that GTFs are required for correct initiation of Pol II transcription, being TFIID usually the first factor to bind DNA by recognizing the TATA box, initiator (Inr) and/or downstream

promoter element (DPE) found in most core promoters. TFIID is a multiprotein complex containing TBP (TATA box binding protein) and approximately 14 TBP-associated factors (TAFs), which are important for promoter recognition rather than activation [12]. After TFIID association with DNA, TFIIA and TFIIB are recruited to stabilize the promoter-bound TFIID. Pol II and TFIIF are then recruited, followed by TFIIE and finally by TFIIH. TFIIH has three enzymatic functions required for transcription: ATPase involved in transcription initiation and promoter clearance, ATP-dependent helicase to unwind the DNA, and CTD kinase. Alternatively, Pol II can also exist as a preassembled holoenzyme complex consisting of Pol II, a subset of GTFs and other proteins. Once PIC is assembled, Pol II escapes from the promoter in a process known as promoter clearance, to start with mRNA elongation. Meanwhile, a scaffold structure composed of TFIID, TFIIE, TFIIH and Mediator remains on the core promoter for subsequent reinitiation of transcription, which is a much faster process than the initial round [13].

1.2 Transcription factors and cofactors

Formation of the PIC is sufficient for a basal level of transcription *in vitro*. However, transcriptional activity is greatly stimulated by transcription factors and coactivators. In the cells, a tight regulation of transcription is in part achieved by transcription factors (activators and repressors) and cofactors (coactivators or corepressors).

1.2.1 Transcription factors

In general, promoter-specific transcription factors bind DNA in a sequence-specific manner upon activation. The recognition sites of transcription factors are usually upstream of the core promoter, either in proximal promoters or enhancers [14]. Depending on the cellular context, these proteins can either activate or repress transcription. Several signaling pathways control the activities of transcription factors [1].

DNA-bound activators are thought to stimulate transcription by recruiting histone-modifying enzymes and remodeling complexes to generate local chromatin decompaction, thus allowing the binding of the transcription machinery; activators also influence transcription by interacting directly with GTFs and Pol II to allow targeting of them to gene

promoters [15]. Conversely, repressors can influence transcription in a negative way either by blocking the binding of a nearby activator, by directly competing for binding to the same site, by establishing a 'closed' chromatin structure or by inhibiting PIC assembly [16]. In some cases, an activator can become a repressor by differential cofactor recruitment.

1.2.2 Transcription cofactors

Typically, cofactors are proteins that regulate transcription without binding directly to DNA; they are rather recruited through protein-protein interactions with a DNA-bound transcription factor. Like transcription factors, transcription cofactors can either stimulate (coactivators) or inhibit (corepressors) transcription by linking transcription factors with GTFs, by modifying chromatin and by stimulating PIC formation (reviewed in [17]). Many transcriptional cofactors are enzymes that directly modulate the chromatin structure by remodeling nucleosomes or by modifying histones (discussed in more detail in sections 2.2 and 2.3 respectively) [1]. A general feature of coactivators is that they can stimulate transcription synergistically.

Mediator is a key coactivator of Pol II, which is absolutely required for both basal and activator-dependent transcription *in vitro* and *in vivo* of almost all Pol II promoters from yeast to humans (reviewed in [18]). Mediator is a multi-protein complex with more than 20 subunits that bridges the interaction between transcription factors at an enhancer and the transcription machinery at the promoter to stimulate the initiation of transcription [19]. But mediator does not only function as coactivator, it can also repress transcription [20].

1.3 Transcriptional regulatory elements

In order to modulate transcription, GTFs and transcription factors bind to regulatory sequence elements on the DNA, including core promoters, proximal promoters, enhancers, silencers and insulators.

1.3.1 Core promoter elements

Core promoter is the minimal portion of the promoter required to properly initiate transcription *in vitro*, since it is the platform for the assembly of transcription PIC. It

typically extends 40-50 bp up- and downstream of the TSS, thus defining the position of the TSS as well as the direction of transcription [21]. To date, seven core promoter elements have been identified, namely TATA box, the initiator (Inr), the downstream promoter element (DPE), the motif ten element (MTE), the downstream core element (DCE), the upstream TFIIB-recognition element (BRE^u) and the downstream TFIIB-recognition element (BRE^d) (reviewed in [22]). The TATA-box, which is recognized by the TBP subunit of TFIID, was the first core promoter element to be described.

1.3.2 Proximal promoters, enhancers, silencers and insulators

The proximal promoter is the region immediately upstream from the core promoter. It constitutes a few hundred bp long region where multiple binding sites for transcription factors can be found. Transcription factors can also bind DNA on enhancers to activate transcription. Enhancers are usually located far away from the core promoter, either up- or downstream of TSS, even in introns (reviewed in [23]). Many different transcription factors, coactivators and chromatin remodeling proteins assemble at enhancers and facilitate the recruitment of Pol II to the promoter of a gene located several kilobases (kB) away by DNA loop formation (Figure 1). Thus, enhancers regulate genes from a distance in a position- and orientation-independent manner (reviewed in [24]).

Silencers, on the other hand, are sequence-specific elements that regulate transcription in a negative manner by binding repressors that prevent Pol II to initiate transcription. As for the enhancers, silencers can be located far away from the TSS.

Insulators are genetic boundary elements that block genes from being affected by the transcriptional activity of neighboring genes by disrupting the communication between a regulatory sequence and a promoter (reviewed in [25]). By doing so, insulators influence transcriptional regulation and global nuclear organization (reviewed in [26]).

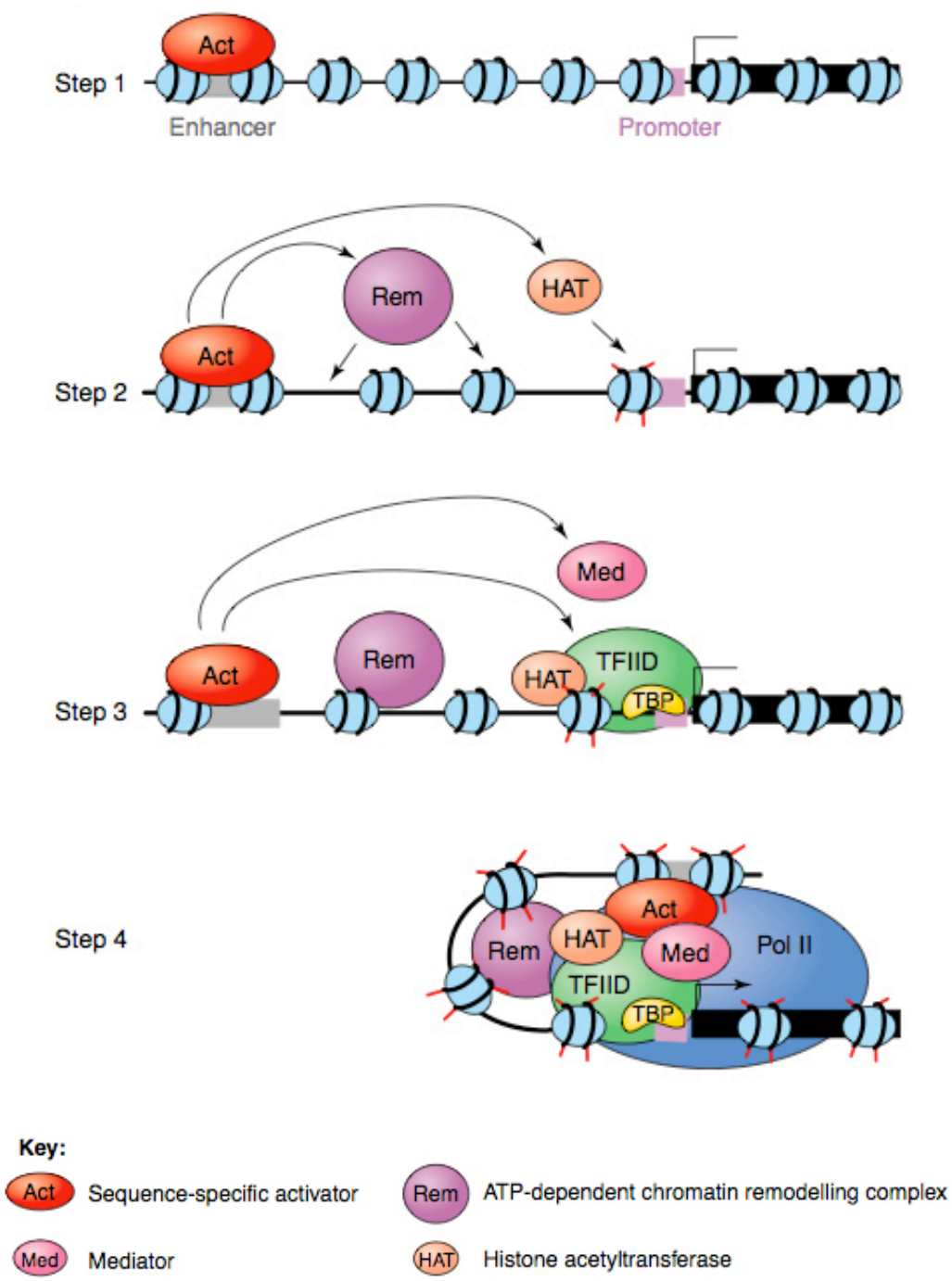


Figure 1. *Classical model of transcription activation in eukaryotes.* The activator (Act) binds to the distal enhancer (step 1) and recruits chromatin remodeling complexes (Rem) and histone acetyltransferases (HAT) to make the chromatin more accessible (step 2). TFIID can then bind to the core promoter to nucleate the assembly of the PIC (step 3). Mediator (Med) then bridges the interaction between the enhancer-bound activator and the transcription machinery at the promoter to recruit Pol II and initiate transcription (step 4) [27].

1.4 Revision to the classical definitions of eukaryotic Pol II transcription

The GTFs are considered 'general' because they were proposed to be required for the transcription of every Pol II dependent gene [28]. However, recent *in vivo* studies have shown that not all GTFs are present in every PIC, in part because certain GTFs are not expressed in every cell type of a given organism [29]. In addition, although TFIID was initially defined as a TATA-binding factor, it seems to act primarily through TATA-less promoters [30]. On the other hand, TBP binding to the TATA box is not a prerequisite for gene expression *in vivo* [31].

Several studies have shown that the core promoter elements may not be as universal as once thought neither. For example, the TATA box has been recently identified as being present in less than 30% of all human and yeast Pol II promoters [30, 32, 33]. Furthermore, a whole class of promoters has been described that contain multiple TSS over a region of 100 bp, referred as broad-peak TSS-containing promoters [34]. They are much more abundant than single peak promoters and their regulation seem to be completely different. While single peak promoters contain usually a TATA box and are involved in the tight regulation of tissue-specific genes, broad peak promoters often harbor CpG islands, are TATA-less and associate with ubiquitously expressed genes. In addition to multiple TSS, alternative promoters of protein-coding genes have been also described, which are clearly separated by several hundred bp [35, 36]. These alternative promoters have been associated with a tight regulation of developmental genes.

Recent *in vivo* studies have shown that Pol II recruitment to DNA is by far more complex than originally described. PIC assembly was initially described to occur at the core promoter. Recent studies have shown that Pol II and GTFs may also bind to enhancer and intergenic regions, though more weakly than to promoters (reviewed in [37]). Binding to enhancer regions has been observed as an early event in gene activation, thus playing a role in transcriptional initiation. Some enhancers have been proposed to function as nucleation centers for the PIC assembly to regulate the timing of gene activation during development, differentiation and the cell cycle (reviewed in [27]). After formation of the PIC at some enhancers, the active PIC would be quickly transferred to the specific promoters when the cell receives the necessary signal. It is still not clear how general this event is, and whether the PIC is transferred from the enhancer to the promoter by DNA looping or by tracking.

Enhancers do not only localize in *cis* up to several hundred kB away from the regulated gene, they can also be confined to another chromosome, thus modulating transcription in *trans* [38]. Inter-chromosomal associations have been described to be involved in processes such as T lymphocyte differentiation, genomic imprinting, X inactivation and expression of odorant receptor in sensory neurons [39].

Several studies have shown that Pol II can accumulate at inactive or lowly expressed genes. For example, chromatin immunoprecipitation (ChIP) assays in *Drosophila melanogaster* revealed Pol II localization to proximal promoter of heat-shock genes already before heat-shock induction [40], which was a surprise since Pol II recruitment was thought to be the limiting step in transcription. Later, Pol II was found to be transcriptionally engaged at these promoters, though arrested after formation of a short nascent RNA molecule of about 25 nucleotides [41]. These findings introduced the concept of 'Pol II proximal promoter pausing', which occurs at a variety of genes, is conserved from yeast to humans, and is thought to be required for full activation of genes (reviewed in [42]). Thus, in addition to PIC formation, mRNA elongation constitutes an important regulatory step of transcription as well.

Genome-wide investigations showing Pol II pausing at proximal promoters have also reported transcription in the opposite direction of the sense RNA, referred to as antisense transcription. Though initially thought to be a rare event, several recent studies have shown that bidirectional transcription is a general feature of eukaryotic transcription [43-48]. Moreover, it correlates with active promoter regions rather than having a random distribution through the genome. However, while sense RNA tends to get elongated, antisense RNA transcription fails to proceed beyond promoters. Whether antisense transcription is a byproduct of sense transcription, or whether it is required for sense transcription is still not clear.

The completion of the Human Genome Project on 2003 allowed the identification and analysis of functional elements in 1% of the human genome by the Encyclopedia of DNA Elements (ENCODE) project [49]. They identified previously unrecognized TSS, many of them hundreds of thousands of bases away from the known TSS. Additional exons from the protein-coding genes were also described, sometimes located thousands of bases away from the gene, sometimes even hidden in another gene. In addition, they found that approximately one forth of the recognized promoters is located at the end of the gene. One of the main

conclusions of this study was that the human genome is pervasively transcribed, since 80% of the bases studied were expressed. The majority of this transcription was shown to take place at non-coding DNA, consistent with the finding that only around 1% of the whole genome is protein-coding DNA [50]. Non-coding RNAs (ncRNAs) are known to regulate gene expression at different levels, including chromatin structure, transcription, RNA processing, RNA stability and mRNA translation (reviewed in [50] and [51]). In addition to ribosomal and transfer RNA, which constitute large part of all ncRNA, other ncRNAs have been identified in the last years. Some examples are small RNAs (small interfering RNA, microRNA and piwi-associated RNA) important for gene expression regulation, small nuclear RNAs involved in splicing of mRNA, *Xist* necessary for inactivation of the second X chromosome in females and telomerase RNA used as template for addition of telomeres [52-56]. The ENCODE project allowed the discovery of numerous novel ncRNAs, whose functions have yet to be determined.

The local environment of a gene such as nucleosome positioning and histone modifications heavily affects the transcription of a gene (reviewed in [57] and [58]). Furthermore, the formation of higher order structures of chromatin and the position of a gene in the nucleus also influences gene expression (reviewed in [59] and [60]). The impact of chromatin structure on transcription will be discussed in the following sections.

2 Chromatin

Genomic DNA has to be efficiently packaged in order to fit into a cell, given that DNA is considerably longer than the diameter of every cell. This compaction is achieved in eukaryotic cells by wrapping DNA around histone proteins, forming so a dynamic structure known as chromatin [61]. These core histones are highly conserved proteins that consists of a globular domain and an unstructured N-terminal “tail” that is subjected to several posttranslational modifications, generating different chromatin structures [58]. The relaxed structure permissive to transcription is called euchromatin and is often associated with active transcription of genes. In contrast, constitutive heterochromatin is tightly packed and inaccessible to transcription factors, thus it is poorly transcribed [62]. There exist an

intermediate state, facultative chromatin, which is condensed and repressed as constitutive heterochromatin, but can decondense to allow transcription under specific conditions (reviewed in [63]).

2.1 Nucleosome

The fundamental subunit of chromatin is the nucleosome, which consists of 147 bp of DNA wound around an histone octamer composed of two copies each of the four core histones (H3, H4, H2A and H2B) [64]. These core histones contain a globular domain involved in histone-histone interactions, and an N-terminal histone tail, which is approximately 15 to 30 amino acids (aa) long and highly modified posttranslationally [65]. A chain of nucleosomes can coil in a regular manner to form a so-called 11-nm fiber (Figure 2). The next level of compaction is the 30-nm fiber, in which linker histone H1 is incorporated and the DNA is furthermore condensed in part due to the neutralization of the DNA charge by the positively charged histone tails [57].

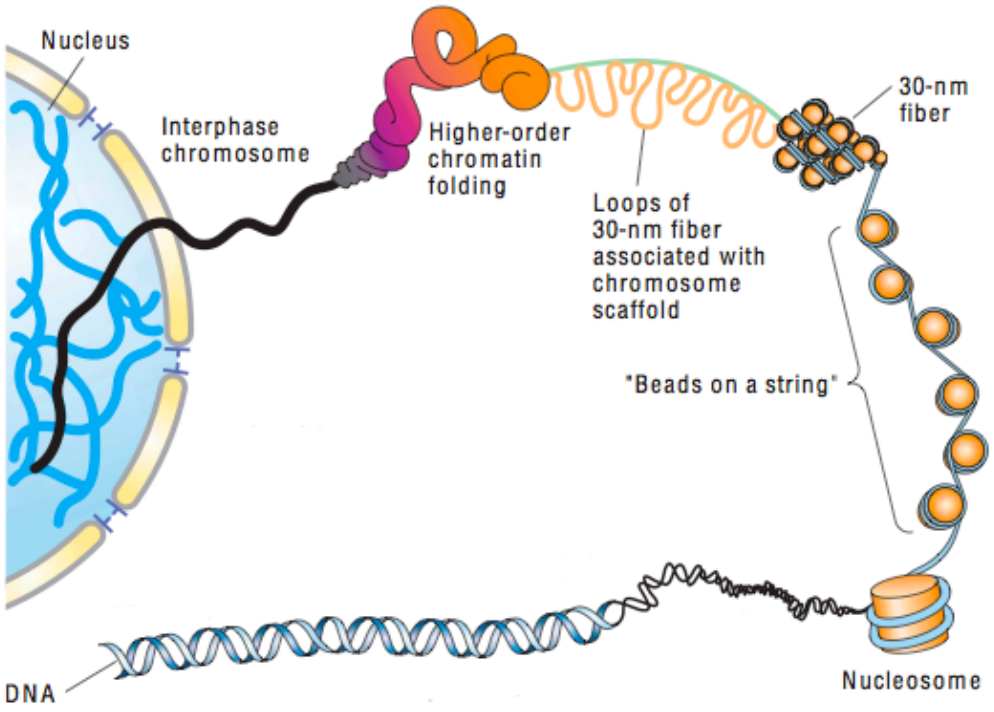


Figure 2. *Chromatin structure*. Schematic representation of the DNA wrapped around eight core histones in the nucleosome, forming "beads on a string" structure. The further coiling in condensed chromatin, the 30-nm fiber, inhibits transcription. 30-nm fibers form loops, increasing condensation yields "higher-order chromatin folding" [66].

In general, chromatin creates a natural barrier for the binding of GTFs and transcription factors to their regulatory sequences on DNA [57]. Consistent with this, several genome-wide studies showed that nucleosome density at promoter regions is typically lower than in the coding region [67-69]. Nevertheless, some transcription factors, such as the glucocorticoid receptor, are able to bind to its DNA recognition sequences even when they are packaged into nucleosomes [70].

The dynamic state of chromatin is mainly achieved by two distinct classes of mechanisms: ATP-dependent chromatin remodeling and posttranslational modifications on histones, which will be discussed in the following sections.

2.2 Chromatin remodeling

Chromatin remodeling complexes use ATP hydrolysis to alter the chromatin structure by disrupting or moving histone octamers along DNA [71]. By doing so, they can render nucleosomal DNA either accessible or inaccessible for regulatory proteins. Chromatin remodeling factors are divided into four main families according to their similarities in their ATPase subunit: SWI/SNF (switch/sucrose nonfermenting), ISWI (imitation of switch), INO (inositol), and CHD (chromodomain helicase/ATPase DNA binding protein) [65]. The ATPases are generally present in complexes with 4 to 12 other protein subunits [72].

2.3 Posttranslational modifications of histones

Both histone tails and globular domains are subject to multiple posttranslational modifications at several sites by many distinct classes of enzymes. These posttranslational modifications have a great impact on processes involved in DNA metabolism such as transcription [73]. Up to date, eight distinct types of modifications have been described on histones, including acetylation [74], methylation [75], phosphorylation [76], ubiquitination [77], ADP-ribosylation [78], sumoylation [79], deimination [80] and proline isomerization [81]. They influence the packaging of chromatin by two different mechanisms: recruitment of effector proteins that bind the modified histone and disruption of contacts between nucleosomes (reviewed in [58]).

Posttranslational modifications are dynamic; they can appear and disappear on chromatin within minutes. Thus, enzymes that remove the modifications have been identified. Methylation of histone tails was long believed to be stable and irreversible, until the discovery of the first demethylase, LSD1 [82]. Many of the enzymes that modify histones have non-histone substrates as well.

Acetylation, methylation, phosphorylation and ubiquitination of histones have been implicated in gene activation, whereas methylation, ubiquitination, sumoylation, deimination and proline isomerization have a role in repression (Table 1).

Table 1 | Chromatin modifications

Mark*	Transcriptionally relevant sites†	Transcriptional role‡
DNA methylation		
Methylated cytosine (meC)	CpG islands	Repression
Histone PTMs		
Acetylated lysine (Kac)	H3 (9, 14, 18, 56), H4 (5, 8, 13, 16), H2A, H2B	Activation
Phosphorylated serine/threonine (S/Tph)	H3 (3, 10, 28), H2A, H2B	Activation
Methylated arginine (Rme)	H3 (17, 23), H4 (3)	Activation
Methylated lysine (Kme)	H3 (4, 36, 79) H3 (9, 27), H4 (20)	Activation Repression
Ubiquitylated lysine (Kub)	H2B (123§/120¶) H2A (119¶)	Activation Repression
Sumoylated lysine (Ksu)	H2B (6/7), H2A (126)	Repression
Isomerized proline (Pisom)	H3 (30–38)	Activation/repression

*The modification on either DNA or a histone.
†Well-characterized sites with regard to genomic location for DNA methylation or residues within histones for PTMs.
‡Whether the epigenetic mark is associated with activation or repression.
§Yeast (*Saccharomyces cerevisiae*).
¶Mammals.

Table 1. *Chromatin modifications.* Shown are the various posttranslational modifications of histones, the transcriptionally relevant sites and the transcriptional outcome. DNA methylation was not a subject of this work. Table taken from [83].

Recent observations suggest that there is no clear division between active and repressive modification states [83]. For example, methylation of lysine 4 of histone H3 (H3K4) usually

correlates with transcriptional activation [84-86]. However, H3K4 methylation within the ribosomal RNA locus can lead to repression of Pol II transcription [87].

2.3.1 Crosstalk between different histone posttranslational modifications

Different histone modifications might influence each other (reviewed in [88]). For example, one histone modification could promote the generation of another. This is the case of H3S10 phosphorylation, which facilitates the binding of Gcn5 and the subsequent acetylation of H3K14 [89]. Conversely, one histone modification could impair the formation of another. Posttranslational modification at lysine residues are a good example for this: since lysines can be subjected to many different types of modifications including acetylation, methylation, sumoylation and ubiquitination, one type of modification excludes the others on the same lysine residue. Furthermore, deimination of histones converts arginines to citrullines, thus preventing arginine methylation [80]. Histone modifications could also direct the loss of particular modifications; for example, methylation of H3K36 promotes deacetylation of H3 and H4 by Rpd3S deacetylase complex in yeast [90]. In addition, crosstalk can also occur between modifications on different histone tails; for example, H2B ubiquitination is required for H3K4 and H3K79 methylation in yeast [91].

2.3.2 The histone code

It is thought that every histone modification leads to a specific biological outcome [58]. This has led to the proposal of the histone code hypothesis, which predicts that the posttranslational modifications of the histone tails, alone or in combination, can be "read" by proteins to modulate transcription and other DNA-template programs [92]. Increasing evidence indicates that the modified histones create binding sites for specific domains that are often associated with enzymes that modify or alter chromatin (Figure 3) [93, 94].

For example, the bromodomain, a conserved motif found in many proteins that modulate chromatin structure, binds to acetylated lysine residues on histone H3 and H4 (see section 3.3) [95]. Furthermore, TAF3, a component of TFIID, binds to H3K4me3 via its plant homeodomain (PHD) finger [96]. H3K4me3 is a hallmark of active human promoters, thus this finding sheds light on why this histone modifications is important for gene activation. In

contrast, methylation of lysine 9 on histone H3 (H3K9me3/me2), a mark of heterochromatin, is recognized by the chromodomain of repressive heterochromatin protein 1 (HP1) [97]. This interaction leads to the structural formation of compacted chromatin, which represses transcription. But effector proteins may also recognize unmodified chromatin. For instance, the PHD finger of BHC80, a component of the LSD1 co-repressor complex, preferentially binds to unmethylated H3K4 compared to its methylated counterparts [98].

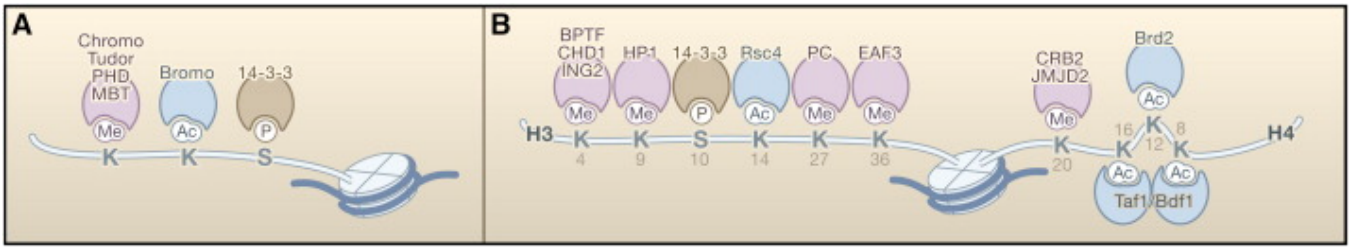


Figure 3. (A) Proteins are recruited to chromatin modifications via specific domains that recognize these modifications. Methylation (Me) is bound by chromo-like domains of the Royal family (chromo, tudor, malignant brain tumor (MBT)) and plant homeodomain (PHD) domains; acetylation (Ac) of lysines (K) is recognized by bromodomains (Bromo), and phosphorylation (P) of serines (S) by a domain within 14-3-3 proteins. **(B)** Some examples of proteins known to interact with modified chromatin via their specific domains: bromodomain PHD finger transcription factor (BPTF), chromodomain helicase DNA binding protein 1 (CHD1), inhibitor of growth family member 2 (ING2), heterochromatin protein 1 (HP1), 14-3-3, remodels the structure of chromatin (Rsc4), polycomb (PC), EPEC adherence factor (EAF3), Crumbs homolog 2 (CRB2), Jumonji 2 (JMJD2) and bromodomain containing 2 (Brd2) [58].

It is important to keep in mind that the histone code is not as strict as the genetic code: one specific posttranslational modification on histone tails does not always correlate with the same outcome [99]. The cellular context plays an important role in determining the functional readout of a posttranslational modification during a specific cellular response to an extracellular or intracellular stimulus.

3 N^{ε}-acetylation of proteins

In contrast to co-translational N-terminal α-acetylation of many proteins, N^{ε}-acetylation is a reversible, posttranslational modification that consists of the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the ε-amino group of a lysine in the target protein (Figure 4) [100]. This reaction is also different from the newly described protein O-acetylation, which competes with phosphorylation [101, 102]. Up to date, only lysines have been

demonstrated to be N^{ε}-acetylated. Because lysine acetylation was first described in histones, the enzymes responsible for the catalysis of acetylation are often referred as histone acetyltransferases (HATs) and the ones performing the reversed reaction as histone deacetylases (HDACs). The dynamic equilibrium of these two classes of enzymes in the cells results in a rapid turnover of acetylation [103].

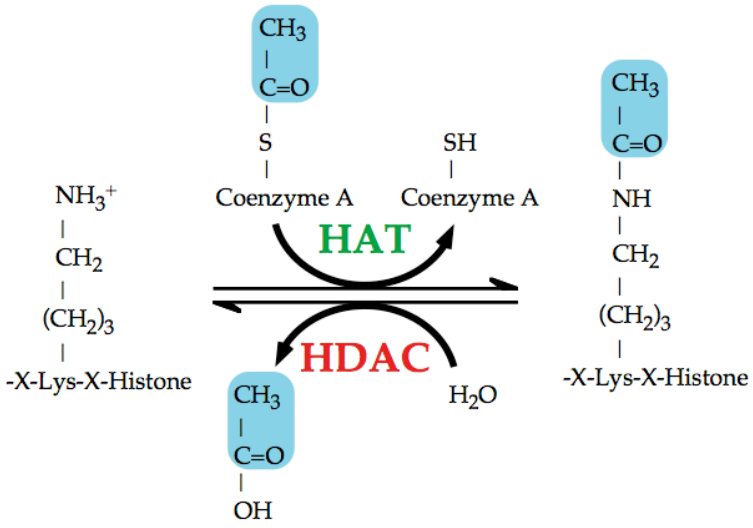


Figure 4. *Acetylation and deacetylation reaction*. Histone acetyltransferases (HATs) transfer the acetyl moiety from acetyl Coenzyme A (acetyl-CoA) to the amino group of internal lysine residues on histone or other non-histone proteins. The reversed reaction is catalyzed by histone deacetylases (HDACs) [100].

Acetylation was first discovered in histones more than 40 years ago, whereas the first HAT was identified only a decade ago [104-106]. Of all the posttranslational modifications, acetylation has the most potential to achieve decompaction of chromatin because it partially neutralizes the positive charge of histones and thus weakens intra- and inter-nucleosomal interactions of the chromatin fiber [107, 108]. It was the first modification of chromatin to be described and to be correlated with increased gene activation [105, 109]. All four core histones (H2A, H2B, H3 and H4) can be acetylated, though H3 and H4 modifications are mainly responsible for gene activation. Deacetylation of histones, in contrast, is associated with repression [100]. Thus, it is no surprise that certain histone deacetylases (HDACs) are part of repressor complexes, while some transcriptional coactivators possess intrinsic histone acetyltransferase (HAT) activity [74, 110].

Fifteen years after histone acetylation was discovered, acetyl lysine residues were found in high-mobility group protein 1 (HMG1) [111]. More importantly, the first non-

histone target of HAT was reported only a decade ago: p53 [112]. Since then, many other non-histone proteins have been described to be acetylated [113]. It is now well established that acetylation is a quite common posttranslational modification in eukaryotes, virus and bacterial proteins [114]. It has been described in numerous proteins, among them more than 80 transcription factors, many other nuclear regulators and several cytoplasmic proteins [113]. In eukaryotic cells, most acetylated proteins are localized to the nucleus. However, this posttranslational modification is also important for regulating different cytoplasmic processes, such as cytoskeleton dynamics, energy metabolism, endocytosis, autophagy and signaling from the plasma membrane [115].

Once a protein is acetylated, its biological function may be affected in different ways. For instance, protein acetylation neutralizes the positive charge of lysines. As a result, the electrostatic properties of the protein are strongly modified, thus influencing the interaction with other proteins, DNA or RNA [113]. Alternatively, lysine acetylation can impair the ability to form hydrogen bonds, which may be important for processes such as oligomerization [116]. Furthermore, in addition to acetylation, lysines are subject to other posttranslational modifications, including methylation, ubiquitination and sumoylation. Thus, any of these modifications would preclude the other ones and their functional consequences. As in histones, acetylation of a lysine residue might affect modification of a neighboring residue in non-histone proteins as well, which let to the proposal of extending the 'histone code' to a more general term such as the 'protein modification code' [115]. The biological function of a protein may be further influenced by interaction with bromodomain-containing proteins (described in more detail in section 3.4).

3.1 Histone acetyltransferases

HATs are enzymes capable of acetylating histones as well as non-histone proteins [117]. The first HAT to be identified was cytoplasmic Hat1 (Histone acetyltransferases 1) from *S. cerevisiae* [118]. The first nuclear HAT was discovered in the ciliate *Tetrahymena thermophila* and was found to be homologous to yeast Gcn5 (general control non-derepressible 5), followed by the rapid identification of HAT activity on several known transcription-related proteins, such as TAF1 ($TAF_{II}250$) and CBP [119-121]. HATs are evolutionary conserved from yeast to humans and present in multimeric complexes involved in transcription as coactivators [122]. In general, HATs have limited specificity by

themselves; the remaining subunits of the multimeric complexes allow the targeting of the HAT complex to the correct substrate and the regulation of the specific activity [123]. Interestingly, multiple HATs have been shown to acetylate the same substrate [114].

HATs can be divided into three main families on the basis of their catalytic subunit: GNATs (Gcn5-related N-acetyltrasnferases), p300/CBP (E1A-associated protein of 300 kDa/CREB-binding protein) and MYST proteins [124].

3.1.1 GNATs

Gcn5, a protein present in all eukaryotes, is the founding member of the GNATs [125]. In yeast, Gcn5 is present in four distinct multiprotein complexes: SAGA (Spt-Ada-Gcn5-acetyltransferase), ADA, SLIK and HAT-A2 [117, 126]. Mammalian PCAF (p300/CBP-associated factor), a structurally similarly protein to yeast Gcn5, is also a member of this family of HATs [127]. Other HATs included in this family are yeast Elp3, Hat1, Hpa2 and Nut1 [117].

3.1.2 MYST

Another group of HATs is the MYST family, named by he founding members: MOZ, Ybf2 (Sas3), Sas2 and Tip60 [74]. Tip60 (Tat-interactive protein, 60 kDa) was the first human MYST member to be identified [128]. In addition to the founding members of this family, new HATs have been identified, including yeast Esa1, *Drosophila* MOF, and human HBO1 and MORF. These enzymes are involved in the regulation of a wide range of cellular processes in diverse organisms, such as transcriptional silencing, cell cycle progression, dosage compensation, transcriptional activation and oncogenic transformation [74].

3.1.3 p300/CBP

p300 and CBP are two different nuclear proteins with high sequence homology and very similar cellular functions, which possess HAT activity [121, 129]. They are able to modify all four histones *in vitro* with little apparent specificity [129], as well as non-histone proteins. In addition, p300 and CBP have been shown to autoacetylate themselves to regulate their HAT activity [130].

These very large proteins (around 300 kDa) are ubiquitously expressed proteins found only in metazoans [131]. p300/CBP are transcriptional coactivators that are able to mediate the communication between at least 40 different transcription factors and the basal transcription machinery [132]. Since they are thought to be present in limiting amounts in the cells, transcription factors have to compete for these pivotal co-activators. Knockout mice for p300/CBP dye at embryonic stage, reflecting how important these proteins have become for higher eukaryotes [133].

3.1.4 Other HATs

In addition to the HATs mentioned above, over 10 other proteins have been described to possess intrinsic HAT activity. However, since they do not contain true consensus HAT domains, they are classified as an 'orphan class' of HAT enzymes [134]. Among these HATs is TAF1, the largest subunit of TFIID. It was shown to contain histone acetyltransferase activity *in vitro*, which may allow the formation of the PIC after acetylating histones at the TATA box [120]. Subunits of TFIIIC, a general transcription factor in the RNA pol III basal machinery, have also been shown to possess HAT activity [135]. The human nuclear receptor coactivators SRC-1 (Steroid receptor coactivator 1) and ACTR (Activator of retinoid receptor) are additional members of the 'orphan class' of HATs [136, 137]. In addition, CLOCK, a circadian rhythm protein, has been described as an acetyltransferase, too [138]. Yeast Rtt109 (regulator of Ty1 Transposition gene product 109) is a member of this family of HATs as well; it promotes genomic stability through the direct acetylation of H3K56 [139].

Several other proteins have been described as members of the 'orphan class' of HATs, including Elp3 (Elongator protein3), Eco1 (Establishment of cohesin 1), CDY (chromodomain on 1 chromosome), and TFIIB [124].

3.2 Histone deacetylases

HDACs were first discovered in 1996 [140]. Mammalian HDACs are divided phylogenetically into four main classes based on their homology to enzymes first described in yeast: class I HDACs are homologues of yeast Rpd3 (reduced-potassium-dependency gene 3), class II HDACs of yeast Hda1 (a subunit of histone deacetylase A complex) and class III HDACs of Sir2 (silent information regulator 2) [141]. The last identified HDAC, HDAC11,

has similar homology to both Rpd3 and Hda1 and is therefore grouped into a separate class; class IV [142, 143]. Class I, class II and class IV form together the 'classical' family of HDACs, whereas class III is also referred to as 'sirtuins'.

3.2.1 'Classical' HDACs

The 'classical' family of HDACs contains 11 members in humans: class I includes HDAC 1, 2, 3 and 8; class II is composed of HDAC 4, 5, 6, 7, 9 and 10; and class IV includes only HDAC11 (depicted in Table 2) [141]. HDAC1, the orthologue of yeast Rpd3, was the first HDAC to be described [140]. Members of the 'classical' HDAC family are conserved from yeast to human and have a quite similar mechanism of action, since they all require zinc ion to hydrolyze the acetyl group [103]. However, additional cofactors are required for HDAC activity, since most recombinantly expressed enzymes are found to be inactive [103]. The 'classical' HDACs are inhibited by trichostatin A (TSA). The majority of HDACs are found in multiprotein co-repressor complexes that act on specific regions of chromatin by deacetylating it, thus repressing transcription [141].

HDAC group	HDAC members	Localization	Expression	Function
Class I (Rpd3)	HDAC1, HDAC2, HDAC3, HDAC8	Nuclear	Ubiquitous	Involved in a variety of functions, such as transcriptional repression and cell differentiation.
Class II (Hda1)	HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10	Nuclear and cytoplasmic	Tissue-specific	Transcriptional repression, microtubule regulation
Class IV	HDAC 11	Nuclear	Tissue-specific	Immune tolerance

Table 2. *'Classical' histone deacetylases*. Adapted from [65].

All class I members are widely expressed proteins [144]. They, together with the class IV member, localize exclusively to the cell nucleus. In contrast to class I HDACs, class II HDACs members are expressed in a tissue-specific manner and described to regulate differentiation and development [144]. They are able to shuttle between nucleus and

cytoplasm in response to certain cellular signals [141]. Some of them are transcriptional repressors that are exported from the nucleus to allow transcriptional activation of genes [110].

3.2.2 Sirtuins

Yeast Sir2 has been implicated in DNA repair, recombination, replication and increased replicative lifespan [145]. Furthermore, Sir2 has been shown to silence transcription at telomeres, mating-type loci and ribosomal DNA (rDNA), through deacetylation of histones [146]. Sir2 is highly conserved from bacteria to higher eukaryotes and has seven homologues in mammals, called sirtuins (SIRT1 to 7), depicted in Figure 4. They share a conserved catalytic core domain of ~250 amino acid (aa) residues in the central part of the proteins [147].

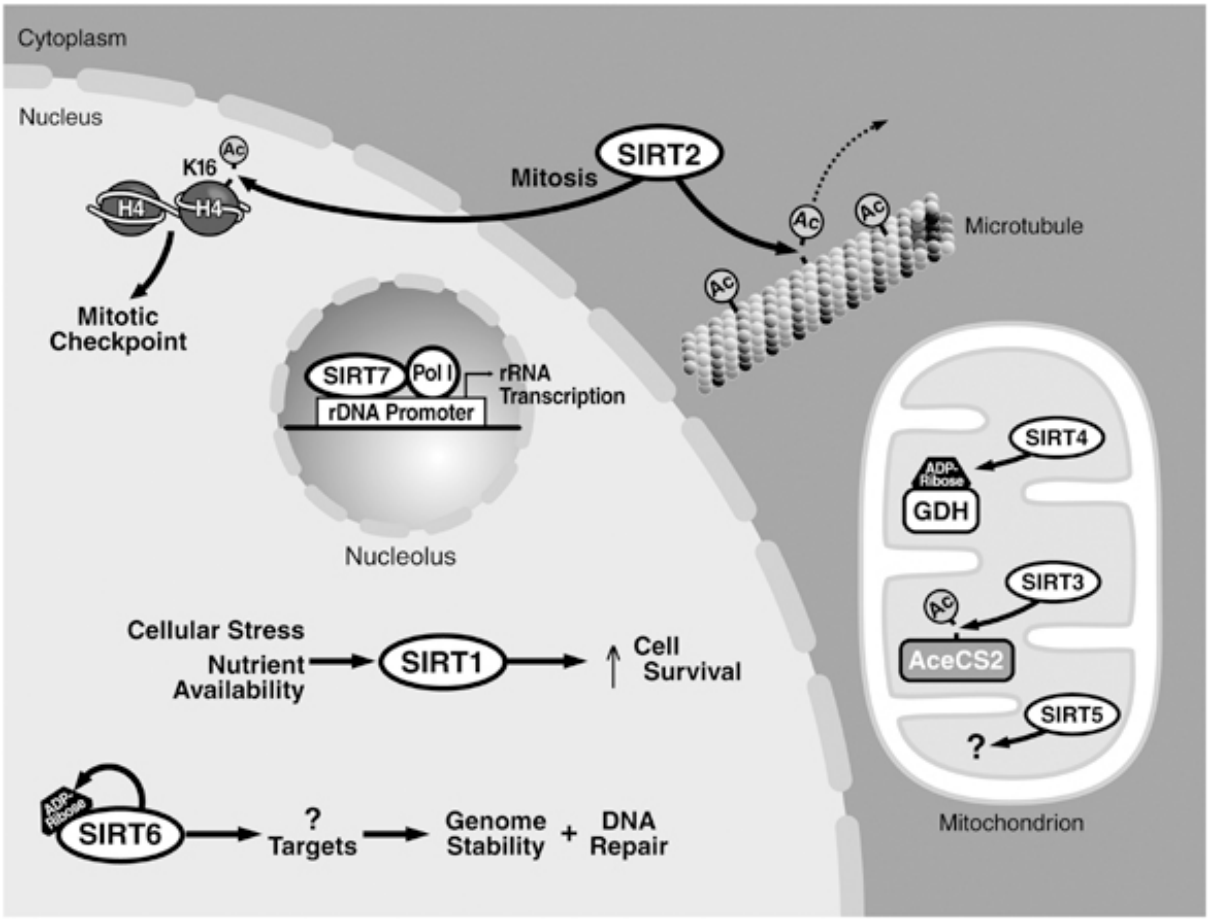


Figure 4. *Sirtuins.* Shown are the cellular localizations, enzymatic activities and targets of the mammalian sirtuins [148]. The newly identified deacetylation target of SIRT6, H3K9, is not depicted in this figure.

In addition to deacetylate proteins, some sirtuins have been described to ADP-ribosylate them. They require nicotinamide adenine dinucleotide (NAD^+) as a cofactor in their deacetylation reaction unlike class I, II and class IV HDACs, which need zinc [146]. This NAD^+-dependency links sirtuin proteins to the metabolic state of cells and helps to regulate sirtuins' activity [149]. During the deacetylation reaction, nicotinamide (Nam) is cleaved from NAD^+ and the acetyl group of the substrate is then transferred to NAD^+, generating the novel metabolite 2'-O-acetyl-ADP ribose (OAADPr) [150]. Since Nam is a byproduct of the reaction, an excess of it in the cells inhibits sirtuins' deacetylase activity. Up to date, all sirtuins have been described to have deacetylation activity except for SIRT4, which is able to ADP-ribosylate proteins, as well as SIRT6, SIRT2 and SIRT3 [151-155].

SIRT1 is the best-characterized member of this family, given its higher homology to Sir2 [153]. As its yeast homologue, SIRT1 has been implicated in the formation of heterochromatin, as well as survival under stress, glucose homeostasis and aging. It is found predominantly in the nucleus, where it deacetylates histones H3K9, H4K16, and H1K26, as well as transcriptional regulators such as TAF_I68, p53, p300, forkhead box O (FoxO) transcription factors and nuclear factor κB (NF-κB) [147, 156-159]. These are only some examples of the growing number of SIRT1 targets.

SIRT2 has been described to regulate microtubule dynamics and possibly cell cycle progression [160-162]. It was originally identified as a cytoplasmic protein, but growing evidence indicates that it can also localize to the nucleus under specific conditions [162-164]. SIRT2 has been described to deacetylate α-tubulin and FoxO1 in the cytoplasm, while histone H4K16 and p300 were identified as its nuclear targets [161, 162, 165, 166].

SIRT3, 4 and 5 are localized to the mitochondria, where SIRT3 and SIRT5 deacetylate several proteins [163, 167]. SIRT4 inactivates glutamate dehydrogenase (GDH) by ADP-ribosylating it, thus regulating the levels of insulin [151]. SIRT6 is a nuclear protein able to ADP-ribosylate itself [152]. Recently, it has been shown to deacetylate H3K9, which modulates telomeric chromatin and represses NF-κB dependent genes [168, 169]. Lastly, SIRT7 is localized to nucleoli, where it activates transcription by RNA polymerase I [170]. p53 has also been described to be deacetylated by SIRT7 [171].

3.3 Bromodomain as acetyl lysine binding module

Acetylation regulates several cellular processes, specially chromatin compaction and gene expression. One way of doing so is by creating docking sites for bromodomain-containing proteins. The bromodomain was initially described as an evolutionary conserved domain of about 110 aa present in several proteins involved in transcriptional regulation, such as the HATs p300, CBP, GCN5, PCAF and TAF1, and other chromatin factors, listed in Table 3 [114, 172]. After the discovery of the specific interaction between the bromodomain of PCAF and acetyl lysines [173], it was clear that this is a module on proteins that binds acetyl lysines.

Many bromodomain proteins are part of multi-protein complexes, which after recruitment to the acetyl lysine execute their biological function. Therefore, bromodomains have important roles in persistent chromatin acetylation by HATs and acetylation-dependent nucleosome assembly and remodeling [114]. In addition, bromodomains bind to acetylated non-histone proteins. The binding specificity of different bromodomains to their specific interaction partners is determined by flanking residues of the acetyl lysine, which are also recognized by bromodomains [174]. In fact, acetyl lysine is necessary but not sufficient for efficient binding by bromodomains [114].

Several bromodomain proteins have two of these modules. Interestingly, sequence alignment have revealed that when two bromodomains are present in the same protein, they share less similarity to each other than to similarly located bromodomains in other related proteins [172]. Thus, different bromodomain subunits may cooperate to recognize distinct acetylated sites on the same target.

Species/ Protein	# bromo	binding to acetyl lysine in	Complex	Biological function
S. cerevisiae				
Gcn5	1	H4 and H2A	SAGA, ADA, HAT2, SALSA	Acetylation, coactivator
Spt7	1	nucleosome	SAGA, SALSA	Coactivator
Swi2/Snf2	1	nucleosome	SWI/SNF	Chromatin remodeling
Sth1	1		RSC	Chromatin remodeling
Rsc1	2		RSC	Chromatin remodeling
Rsc2	2		RSC	Chromatin remodeling
Rsc4	2	H3K14	RSC	Chromatin remodeling
Bdf1	2	H4	TFIID interaction, SWR	Anti-silencing
Bdf2	2		TFIID interaction	
TBP7/YTA	1			
Mammals				
GCN5	1	H4K8	STAGA, TFTC	Acetylation, coactivator
PCAF	1	H3, H4, Tat	PCAF	Acetylation, coactivator
p300	1	MyoD, STAT3		Acetylation, coactivator
CBP	1	histones, MyoD, c-Myb, p53		Acetylation, coactivator
TAF1	2	H3, H4, p53	TFIID	Transcription initiation
MLL	1		ALL-1 complex	Histone methylation
MLL2	1			Homolog of ALL-1
hASH1	1			Histone methylation
Brm	1		BAF (hSWI/SNF)	Chromatin remodeling
BRG1	1		BAF, PBAF, NUMAC	Chromatin remodeling
Polybromo	6		PBAF	Chromatin remodeling
BAZ1A	1		huCHRAC, hACF/WCRF, hNURF	Chromatin remodeling
BAZ1BF	1		WICH	Chromatin remodeling
BAT2A	1		NoRC	Chromatin remodeling
BAZ2B	1			
BPTF	1		hNURF	Chromatin remodeling
Brd2	2	H4K12	Mediator	Coactivator
Brd3	2	chromatin		Coactivator
Brd4	2	H3, H4, p65		Coactivator
Brd5	2	chromatin		
TIF1α	1		HP1 interaction	Corepressor
TIF1β	1		HP1 interaction	Corepressor
TIF1γ	1		HP1 interaction	Corepressor
BR140	1			
Brd1	1			
Brd7	1			Transcr. coregulator
Brd8	1		Tip60	Coactivator
WDR9	2			Coactivator
BS69	1			Corepressor
PRO2000	1			
CECR2	2			
PRKCBP1	1			

Table 3. *Bromodomain proteins in yeast and mammals.* Adapted from [114].

4 NF-κB family of transcription factors

Cells have to continually adapt to changes in their environment. One way to achieve this is by activating inducible transcription factors that modify the pattern of gene expression in the cells. One well-known example of inducible transcription factors is nuclear factor κB (NF-κB), which regulates the expression of a large number of target genes involved in several cellular processes such as immune and inflammatory response, apoptosis, cell proliferation, cell differentiation and survival [175]. Consequently, it is not surprising that a deregulation of this transcription factor would lead to several pathologies, such as inflammatory diseases, neurodegenerative disorders and cancer [176, 177]. In addition, NF-κB p65 subunit knockout mice dye at embryonic day 15.5 by massive degeneration of the liver, strengthening the fact that NF-κB is a key factor in the regulation of normal cellular events [178].

4.1 NF-κB family members

First discovered 23 years ago [179], NF-κB is an ubiquitously expressed family of proteins found in every mammalian cell. It is conserved from the phylum Cnidaria to humans, although it is notably absent in yeast and *C. elegans* [180]. Three Rel proteins have been described in *Drosophila* called Dorsal, Dif and Relish; thus, NF-κB is an ancient signaling pathway and the major mediator of the immune system [181].

The mammalian NF-κB family consists of five members depicted in Figure 5A: p65 (also known as RelA), c-Rel, RelB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2) (reviewed in [182]). All NF-κB subunits share a highly conserved Rel Homology Domain (RHD) at the amino terminus of the protein that is approximately 300 aa long. This domain is important for DNA binding, dimerization of the family members, interaction with the inhibitor of NF-κB proteins (IκB) and nuclear localization. In addition, p65, c-Rel and RelB contain a C-terminal transactivation domain (TAD), required for induction of target genes. The other two members, p105 and p100, are the precursors of p50 and p52, respectively. These long proteins have to be processed by proteosome-mediated partial proteolysis to become active by loosing the ankyrin repeats at the C-terminal region of the proteins. While p100 is processed to p52 upon stimulation and activation of the NF-κB pathway, the majority of p50 is constitutively generated during translation without having p105 as precursor [183].

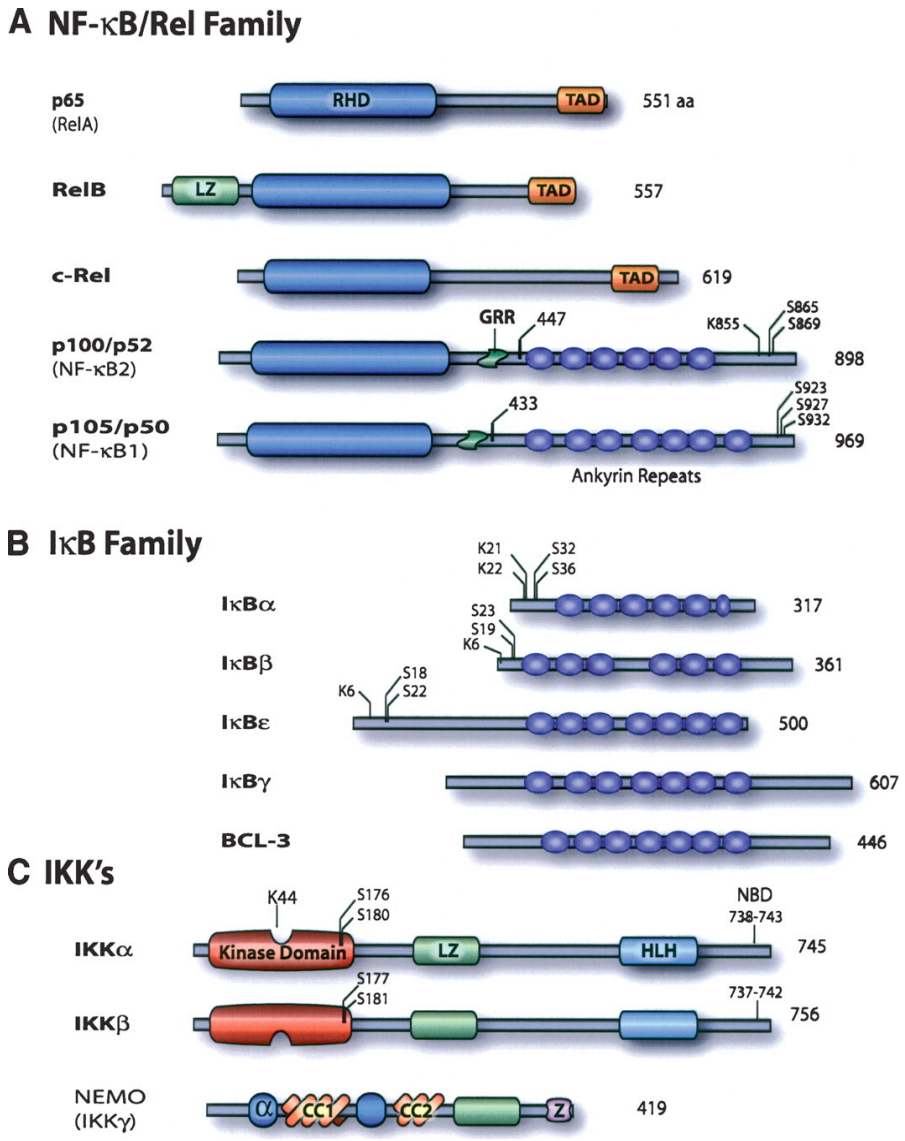


Figure 5. *Schematic representation of NF-κB, IκB and IKK proteins*. Members of the NF-κB **(A)**, IκB **(B)**, and IKK **(C)** family are shown. The number of amino acids is shown on the right. Presumed sites of cleavage of p100 and p105 are indicated. In addition, phosphorylation and ubiquitination sites on p100, p105 and IκB proteins are shown. (RHD) Rel homology domain, (TAD) transactivation domain, (LZ) leucine zipper domain, (GRR) glycine-rich region, (HLH) helix-loop domains, (Z) zinc finger domain, (CC1/2) coiled-coil domains, (NBD) NEMO-binding domain, (α) α-helical domain. Picture taken from [182].

NF-κB family members form homo- or heterodimers with each other, which can then bind to DNA. Up to date, 12 distinct combinations have been described. The most abundant dimer is the one composed of p65 and p50. RelB does not form homodimers, but it can dimerise with p50 and p52. Homodimers of p50 and p52 are unable to induce gene expression because they lack a transactivation domain, and are found associated to silenced genes in unstimulated cells [184].

4.2 NF-κB signaling pathways

There are two well-described signaling pathways that lead to NF-κB activation: the canonical or classical pathway and the non-canonical or alternative pathway, both depicted in Figure 6 (reviewed in [185]). The canonical pathway is induced by pro-inflammatory cytokines such as tumor necrosis α (TNFα) and interleukin-1β (IL-1β), as well as viral and bacterial products.

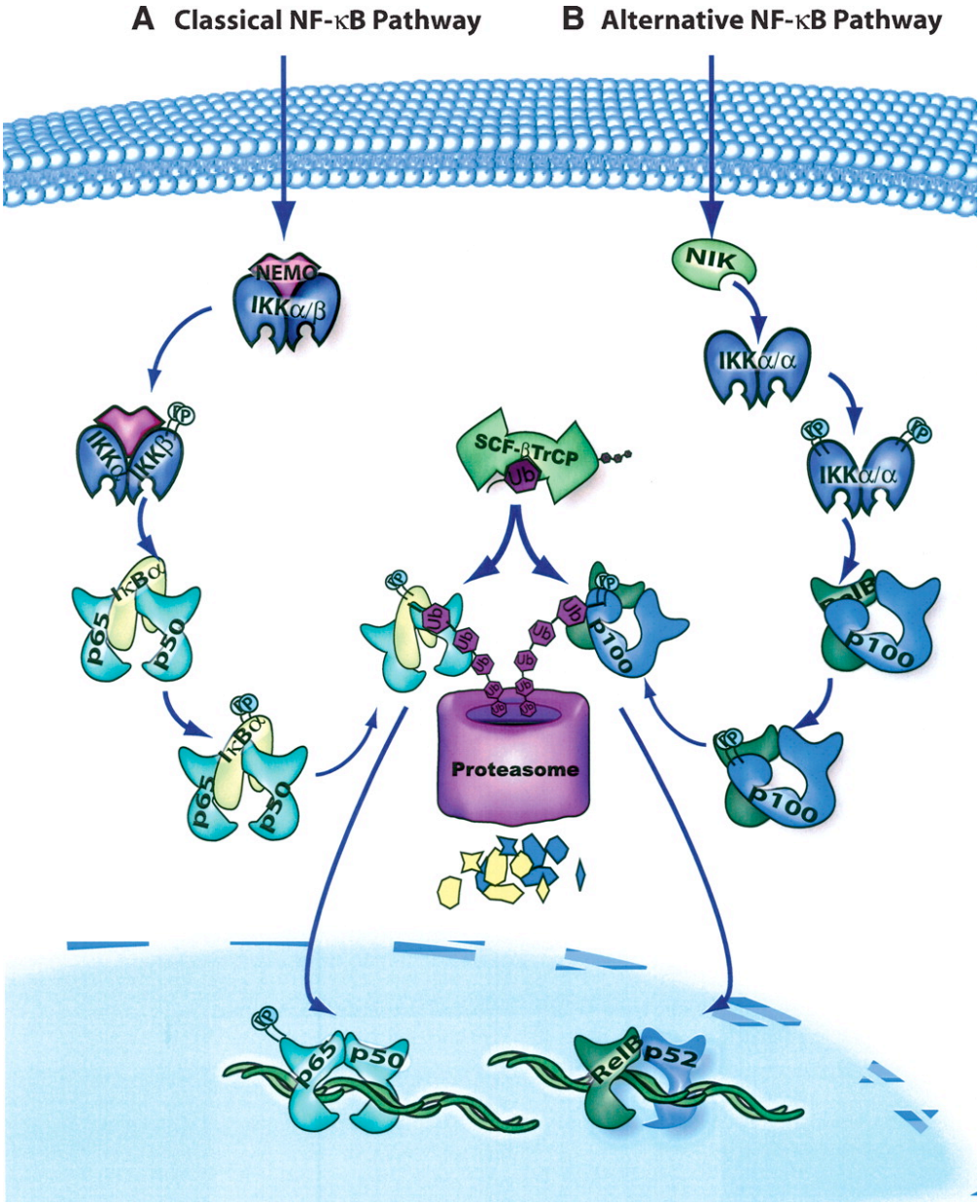


Figure 6. *The classical and alternative NF-κB pathway*. A simplified schematic representation of the two main signaling pathways of NF-κB, the classical **(A)** and the alternative **(B)** pathways. See text for detailed discussion. Picture taken from [182].

In most unstimulated cells, NF-κB dimers containing p65 or cRel reside in the cytoplasm in an inactive form bound to IκBα or IκBβ [175]. IκB proteins possess multiple ankyrin repeat domains and bind to different combinations of NF-κB transcription factors, shown in Figure 5. The prototypical IκB protein is IκBα, which preferentially binds p50/p65 heterodimers. This binding masks the nuclear localization signal (NLS) found in p65, leading to sequestration of the transcription factor in the cytoplasm. Nevertheless, a fraction of the cellular NF-κB/IκBα complexes shuttles between the nucleus and the cytoplasm due to the unmasked NLS of p50 and nuclear export signal (NES) of IκBα.

Once a cell is stimulated, the IκB kinase (IKK) complex is activated and phosphorylates the IκB proteins at specific residues (serine 32 and 36 in IκBα). This event leads to ubiquitylation and subsequent degradation of IκBs by the 26S proteosome, which results in unmasking of the NLS present in p65, rapidly shifting the balance towards translocation of NF-κB to the nucleus. There it binds to specific sequences in promoter or enhancer region of genes, regulating so their transcription by recruiting co-activators and co-repressors. One of the first genes to be induced is IκBα, generating a negative feedback loop: NF-κB is redistributed to the cytoplasm by the newly synthesized IκB protein [186]. Thus, in most cases, NF-κB is transiently activated. Though, since several transcription factors such as p53, c-myc and HIF-1α (Hypoxia-inducible factor 1, alpha subunit) are among its target genes, NF-κB can indirectly influence the expression of many more genes than just its direct targets [187-189]. In the presence of continual inducer, NF-κB activation is cyclical, with nuclear NF-κB binding to DNA appearing and disappearing over time.

The IKK complex consists of two catalytic subunits, IKKα and/or IKKβ, and the regulatory subunit NEMO (NF-κB essential modulator, also known as IKKγ), shown in Figure 5C. IKKβ is mainly implicated in the canonical pathway, while IKKα homodimers are required for the non-canonical one, where they phosphorylate p100 upon stimulation, resulting in its proteosomal processing to p52, depicted in Figure 6 [190]. This leads to liberation of RelB/p52 dimers that were sequestered in the cytoplasm by p100 [191]. The non-canonical pathway is mainly triggered by lymphotoxin-β (LTβ), B-cell activating factor (BAFF) and CD40, resulting in a transcriptional response with a much slower kinetics than the one from the canonical signaling pathway. While in the canonical pathway the induced genes play a role in the activation and regulation of the immune response, the non-canonical

pathway mainly regulates B-cell maturation and secondary lymphoid tissue formation. The two pathways may activate overlapping set of genes, although there are also specific genes [192, 193].

NF-κB can be activated through a less-known pathway, which is independent of IKK complex activation but still requires phosphorylation-dependent degradation of IκBα [194, 195]. DNA damaging inducing agents such as UV irradiation and doxorubicin can trigger this third pathway.

NF-κB signaling pathways can be activated by hundreds of stimuli such as cytokines, Lipopolysaccharides (LPS), growth factors and stress inducers [196]. As an ultimate result, NF-κB induces the expression of a large number of target genes in order to cope with the changing environment. Up to date, around 300 target genes have been described in mammalian genomes [196], (www.nf-kb.org). The set of genes that are regulated by NF-κB differ depending on the cell type and the inducing stimulus [197]. How does NF-κB mediate distinct cellular responses depending on the stimuli and the physiological context? There is no doubt that this can only be achieved through a tight regulation of NF-κB transcriptional activity. A key observation is that not all cell types are able to respond equally to a given stimulus, either because they miss the required receptor or the necessary signal transduction mechanism [198]. In addition, the expression of individual NF-κB subunits change in different types of mammalian cells, and this expression can be altered by environmental and developmental signals [199]. The specificity achieved during transcription is discussed in more detail in the next section.

4.3 NF-κB-dependent transcription

4.3.1 κB site

NF-κB binds to chromatin in a sequence-specific manner to so called κB sites. The sequence of the canonical κB site for p65 is 5' GGGRNNYYCC 3' (R = purine, N = any base and Y = pyrimidine) [179]. It has been proposed that κB sites are often clustered and located in the proximal promoter. Nevertheless, there is more and more evidence that functional κB sites can also be located in intronic regions. In agreement with this, recent genome-wide studies have shown that only close to one third of p65 recruitment to DNA occurs within 5 kB

upstream of transcription start site (TSS) [200, 201]. κB sites are also present in some viral promoters such as human immunodeficiency virus type 1 (HIV-1) and cytomegalovirus (CMV) [202, 203]. Thus, if NF-κB is activated by a virus infection, it can transactivate the viral promoters as well as the host promoters, resulting in an enhanced viral transcription.

Many studies have shown that the κB site sequence is highly degenerate, specially in the 3' half of the site, and that some κB sites that deviate from the consensus sequence can anyway bind NF-κB dimers with high affinity [204]. The κB sites in many promoters are conserved throughout evolution, suggesting that the sequence plays an important role in selectivity. Whether κB site sequence determines which dimer will preferentially bind is still under debate. Initial *in vitro* studies suggested that this would be the case [205]. Nevertheless, with the exception of some cases, most κB sites do not show any clear specificity for a given dimer [206, 207]. In cells lacking individual NF-κB family members, no obvious correlation between κB site sequence and specific dimers have been found, though different NF-κB target genes have distinct requirements for NF-κB proteins [208]. Thus, promoter context rather than just the κB site sequence is important to accomplish specificity.

Despite showing little relevance for dimer specificity, κB sites can influence which cofactor will interact with the bound NF-κB dimer [209]. In order to maximize the contacts with DNA, dimers adopt somehow different conformations depending on the κB site sequence, which influences the ability to interact with specific transcriptional co-regulators [210]. In addition, NF-κB binding to DNA is a very dynamic process which allows rapid exchange of dimers to fine-tune gene expression [211-213]. For example, ChIP-on-Chip experiments in monocytes have shown that p50 and p52 are recruited to a significant number of target genes in unstimulated cells. Upon LPS stimulation, other NF-κB subunits bind to several of these and other genes and, leading to an increase in RNA pol II occupancy and gene expression [213].

κB sites are not only located at the promoter region of target genes, but also on enhancers. For example, p65/p50 heterodimers have been shown to bind to an enhancer located in the second intron of the murine *Mnsod* gene upon TNFα stimulation, necessary for proper expression of *Mnsod* [214]. Another example of NF-κB binding to an enhancer element is at the *IFN-β* gene. In response to virus infection, NF-κB and other transcriptional

regulators bind to the enhancer located immediately upstream of the core promoter of *IFN-β* to form a multi-protein complex known as the *IFN-β* enhanceosome [215]. This coordinate assembly is required to allow RNA pol II binding to the core promoter and initiation of transcription. A recent study shows that NF-κB first binds to at least one of the three identified distinct genetic loci to subsequently bind to the *IFN-β* enhancer to nucleate enhanceosome assembly via interchromosomal associations [216]. Furthermore, NF-κB bound to the enhancer of murine *CD80* gene has been shown to nucleate PIC assembly at this distal enhancer; PIC was then brought to within spatial proximity of the TSS by DNA looping to initiate transcription [217].

The crystal structure of RHD p50/p65 heterodimer bound to a κB site reveals an unusual conformation which resembles a butterfly, with the wings connected to a cylindrical body of DNA, shown in Figure 7 [210]. While the C-terminal domain of the two monomers interact with each other to allow dimerization, the DNA is wrapped around by the N-terminal domains in a way that positioning of the κB site into a nucleosome would constitute a sterical hinder.

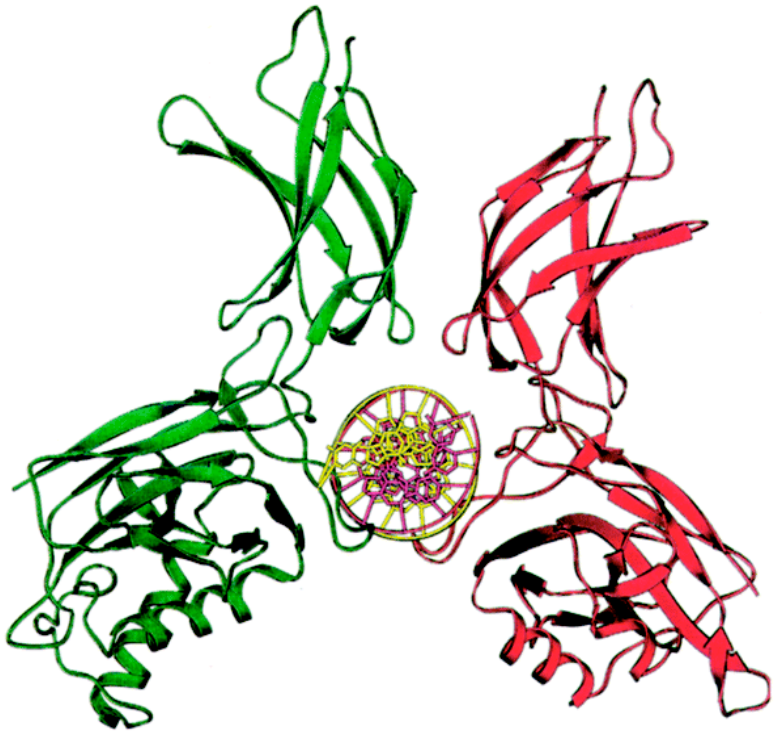


Figure 7. *NF-κB conformation bound to DNA*. The structure of the Rel Homology Domains (RHD) of p50/p65 heterodimers bound to Ig-κB DNA sequence. p50 is depicted in green, p65 in red, the top strand of the DNA is in pink and the bottom strand is in yellow [210].

4.3.2 Impact of chromatin structure on NF-κB-dependent transcription

An additional level of transcriptional regulation is achieved by the chromatin environment surrounding the κB sites of the induced genes. An *in vitro* study shows that the affinity of NF-κB for its response element is not influenced by nucleosome positioning [218]. The situation might be different *in vivo,* if posttranslational modifications of histone tails and folding of chromatin into higher-order structures are considered as possible obstacles to NF-κB binding. Interestingly, studies in macrophages have shown that NF-κB target genes can be divided into two groups depending on how fast they are induced. The first group of genes has constitutively and immediately accessible promoters resulting in fast gene expression while the other group has promoters that require stimulus-induced chromatin modification prior to gene induction [219]. Moreover, it has been shown in LPS-stimulated macrophages that chromatin-remodeling complexes are required for the induction of secondary response genes and primary response genes with delayed kinetics, but not for rapidly induced primary response genes [220]. These could be explained by at least two ways: either NF-κB binds to its response elements regardless of nucleosome positioning and then mediates the removal of inhibitory nucleosomes to allow the transcription machinery to bind, or chromatin-remodeling complexes are first needed to increase the κB site accessibility.

In any case, when p65 enters the nucleus it binds to a broader range of target genes promoters than those whose transcription is being regulated [200, 212]. Thus, although binding of NF-κB to chromatin is required for the activation of target genes, it is not sufficient to drive transcription. Indeed, NF-κB does not function alone, but it often cooperates with several other transcription factors to control gene transcription, such as Sp1 [221], activator protein 1 (AP-1), interferon regulatory factors (IRFs) [222] and E2F1 [201]. The different transcription factors are present depending on cellular context and co-stimulatory signals. This is strengthened by genome-wide mapping studies showing that around 50% of the p65 binding sites do not contain a canonical κB site [200, 201], meaning that either p65 binds to a yet unidentified sequence or that it binds trough interaction with other proteins that associate with DNA.

4.3.3 Cofactors of NF-κB in transcription

NF-κB has been shown to interact with an increasing number of cofactors that can either activate (coactivators) or repress (corepressors) gene expression.

In unstimulated cells, NF-κB target genes are basally repressed by p50 or p52 homodimers bound to κB sites, which recruit co-repressor complexes. More specifically, p50 homodimers have been reported to associate with HDAC1 at DNA, resulting in suppression of NF-κB transcription in unstimulated cells [223]. Other studies have shown that p50 homodimers can tether other repressor complexes at the DNA consisting of SMRT (Silencing mediator of retinoic acid and thyroid hormone receptors) and HDAC3 or N-CoR (Nuclear receptor corepressor) and HDAC3 [224-226]. Upon stimulation of cells, these corepressor complexes are exported from the nucleus to allow transcription to occur, a process known as derepression. In addition, HDAC3 has been reported to repress NF-κB dependent transcription by directly deacetylating p65 after entry into the nucleus, resulting in termination of the NF-κB response [227]. HDAC1 and HDAC 2 have been shown to interact with p65 in the nucleus of unstimulated cells, resulting in a repression of basal as well as post-induction gene expression [228].

SIRT-1 has been described as a potent NF-κB inhibitor, in part, by deacetylating p65 at lysine 310 [159]. Furthermore, SIRT6 has been recently identified as a corepressor of NF-κB for a subset of genes [169]. Additional reported repressors of NF-κB transcription are MYBBP1a (Myb binding protein 1a) [229] and copine-I [230].

Some of the identified NF-κB activators are p300/CBP [231, 232], PCAF [233], SRC-1 [234], TBP and TFIIB [235], TAF4b [236], TAF1 [237], Coactivator-associated arginine methyltransferase 1/ Protein arginine N-methyltransferase (CARM1/PRMT4) [238], Poly ADP-ribosylpolymerase 1 (PARP1) [239], RNA helicase A [240], P-TEFb [241] and Brd4 (Bromodomain containing 4) [242]. The homologous proteins p300 and CBP were found to interact with p65 in response to TNFα and to increase NF-κB reporter gene expression [231, 232]. These two large multi-domain proteins are known to bridge the interaction between several transcription factors and components of the basal transcription machinery (reviewed in [243]). In addition, p300/CBP possess HAT activity and p300 have been reported to acetylate p65, which modulates NF-κB transcriptional activity [244, 245].

PARP1 directly interacts with p300 and synergistically coactivates NF-κB-dependent transcription [239]. This is dependent on p300-mediated acetylation of PARP1 [246].

Another coactivator of NF-κB is CARM1, which belongs to the family of protein arginine methyltransferases. CARM1 has been reported to be involved in transcriptional activation and mRNA stability/degradation through methylation of histones as well as several non-histone proteins [247]. This coactivator was shown to be required for the proper expression of a subset of NF-κB target genes upon TNFα or LPS stimulation [238].

P-TEFb, which consists of kinase CDK9 and cyclin T1, cyclin T2 or cyclin K, has also been described as an NF-κB coactivator. P-TEFb has been described to phosphorylate Pol II CTD, which is required for the elongation of mRNA [248]. To achieve this, P-TEFb is recruited to gene promoters through interaction with Brd4 [249], which is a bromodomain-containing protein known to interact with various proteins to modulate transcription. P-TEFb has been shown to associate with NF-κB at the promoter of a subset of NF-κB dependent genes to stimulate transcriptional elongation by Pol II [241]. Interestingly, a recent study shows that Brd4 binds acetylated p65 through its two bromodomains resulting in increased transcription of a subset of genes [242]. Thus, Brd4 is thought to bridge the interaction between P-TEFb and acetylated p65 at the promoter of specific genes.

In addition to all the aspects mentioned above, specificity of target genes is further achieved by posttranslational modifications of NF-κB, which may affect the interaction with transcriptional coactivators and corepressors [250, 251].

4.4 Regulation of p65 by phosphorylation and acetylation

As histones and many other non-histone proteins, p65 can also be posttranslationally modified, resulting in an alteration of many of its biological functions. The possible modifications of p65 described until now are phosphorylation, acetylation, nitration, oxidation, proline isomeration, ubiquitination and methylation [250]. Many of these modifications are likely to vary between different cell type and stimuli. This section will focus on phosphorylation and acetylation of p65.

p65 is phosphorylated at serine 276 by protein kinase A (PKA) in response to LPS [252] and MSK1/2 (mitogen- and stress-activated protein kinase 1/2) upon TNFα [253]. Other serine targets of phosphorylation are 536 by IKKα, IKKβ and RSK1 (Ribosomal S6

kinase 1); 468 by GSK3β (Glycogen synthase kinase 3β), IKKε and IKKβ [254-256]; 529 by casein kinase 2 (CK2) [257] , and 311 by PKCζ [258], which are reviewed elsewhere [250]. Threonine 435 has also been reported to be phosphorylated at p65 by an unidentified kinase; while dephosphorylation of this site by protein phosphatase 4 (PP4) occurs in response to cisplatin treatment [259]. From all these phosphorylation events, the ones on serine 276 and 536 are required to allow the interaction between p65 and the transcriptional coactivators p300/CBP [260].

Several lysines of p65 are subject to acetylation, mainly by p300/CBP. The Greene lab described acetylation at lysines 218, 221 and 310 by p300/CBP [244]. They showed that while acetylation of lysine 221 enhances DNA binding and, along with lysine 218, impairs interaction with IκBα; acetylation at lysine 310 is required for full transcriptional activity. In contrast, the Benkirane group reported that acetylation of p65 on other lysines (122 and 123) by both p300 and PCAF reduces DNA binding and thus promotes removal from DNA [261]. We have identified two additional acetyl acceptor sites upon TNFα stimulation, namely lysines 314 and 315, and confirmed lysine 310 acetylation [245]. Acetylation on these three lysines regulates the activation or repression of specific subset of genes, therefore providing specificity to NF-κB-dependent gene expression.

Since phosphorylation of p65 on either serine 276 or 536 is needed to allow interaction with p300, it is also required for the subsequent acetylation at K310, thus leading to a crosstalk between these two posttranslational modifications [262, 263]. Because p300/CBP are essential coactivators of both p53 and p65, these two transcription factors compete for p300/CBP [264]. Interestingly, IKKα has been recently reported to phosphorylate CBP in a stimulus-dependent manner, which leads to an increased binding of CBP to NF-κB, resulting in p65 acetylation and NF-κB-mediated cell growth by activation of anti-apoptotic genes [265].

p65 can in turn be deacetylated by SIRT1 and HDAC3, which has been correlated with termination of the NF-κB response [159, 227, 261].

5 Aim of this thesis

NF-κB is an inducible transcription factor known to regulate several cellular processes, including the immune response, apoptosis and cellular proliferation. To do so, it modulates the expression of specific genes selected from the more than 300 possible target genes. Because NF-κB has a pivotal role in the regulation of diverse cellular processes, a tight regulation of its activity is required. Posttranslational modifications, such as acetylation, are known to influence the activity of NF-κB. The aim of this thesis was to elucidate how p65 acetylation affects NF-κB-driven transcription.

RESULTS

6 Published research articles

6.1 Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65

Authors: Christine Buerki, Karin M. Rothgiesser, Taras Valovka, Heather R. Owen, Hubert Rehrauer, Monika Fey, William S. Lane and Michael O. Hottiger

Journal: Nucleic Acids Research, Vol. 36, No. 5, March 2008

Contribution: Planning and performance of the necessary experiments to show p65 acetylation by p300 in cells, interaction between endogenous p300 and p65 in the different complemented cell lines, cell viability after TNFα treatment, gene expression analysis by real-time RT-PCR for a number of genes (*Ccl-20, Ifi-44, Gbp-2*) and of Ccl-7 protein levels. Helping in the experiment describing p65 acetylation at lysine 310 *in vivo* in response to TNFα stimulation, as well as in the analysis of results and the preparation of the figures and the manuscript.

6.2 Deacetylation of p65 by cytoplasmic SIRT2 regulates NF-κB

Authors: Karin M. Rothgiesser, Susanne Waibel, Bernhard Lüscher and Michael O. Hottiger

Journal: Manuscript submitted

Contribution: Planning and performance of all the experiments except cloning of the HA-SIRT2 wild type and mutant constructs. Analysis of all the results and preparation of the figures and the manuscript.

6.3 CARM1 but not its enzymatic activity is required for transcriptional coactivation of NF-κB-dependent gene expression

Authors: Sandrine Jayne, Karin M. Rothgiesser and Michael O. Hottiger

Journal: Manuscript submitted

Contribution: Participation in the generation of custom microarrays specific for the study of NF-κB-dependent gene expression and standardization of this technique for our laboratory.

Published online 7 February 2008 *Nucleic Acids Research, 2008, Vol. 36, No. 5* ***1665–1680***
doi:10.1093/nar/gkn003

Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65

Christine Buerki[1,], Karin M. Rothgiesser[1], Taras Valovka[1], Heather R. Owen[1], Hubert Rehrauer[2], Monika Fey[1], William S. Lane[3] and Michael O. Hottiger[1,*]

[1]Institute of Veterinary Biochemistry and Molecular Biology, [2]Functional Genomics Center Zurich, University of Zurich, 8057 Zurich, Switzerland and [3]Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology, Harvard University, Cambridge, MA 02138, USA

Received September 25, 2007; Revised December 17, 2007; Accepted January 5, 2008

ABSTRACT

Nuclear factor kappaB (NF-κB) plays an important role in the transcriptional regulation of genes involved in immunity and cell survival. We show here *in vitro* and *in vivo* acetylation of RelA/p65 by p300 on lysine 314 and 315, two novel acetylation sites. Additionally, we confirmed the acetylation on lysine 310 shown previously. Genetic complementation of RelA/p65−/− cells with wild type and non-acetylatable mutants of RelA/p65 (K314R and K315R) revealed that neither shuttling, DNA binding nor the induction of anti-apoptotic genes by tumor necrosis factor α was affected by acetylation on these residues. Microarray analysis of these cells treated with TNFα identified specific sets of genes differently regulated by wild type or acetylation-deficient mutants of RelA/p65. Specific genes were either stimulated or repressed by the acetylation-deficient mutants when compared to RelA/p65 wild type. These results support the hypothesis that site-specific p300-mediated acetylation of RelA/p65 regulates the specificity of NF-κB dependent gene expression.

INTRODUCTION

The inducible transcription factor family nuclear factor κB (NF-κB) consists of dimeric proteins involved in many diverse processes such as immune and stress responses and the opposing processes of proliferation and apoptosis (1–3). NF-κB is induced in almost all cell types by different extracellular stimuli causing the activation of an enormous array of target genes (4). Thus, it is not surprising that the specificity of NF-κB responses is very important for the fate of a cell. It has been shown that abnormal NF-κB activity, which is not always associated with genetic alterations, plays a role in different inflammatory diseases and cancer (5–7).

NF-κB specificity is regulated at different levels in the cell (8). One level of regulation is the selective activation of distinct NF-κB complexes after induction by diverse stimuli. In mammals there exist five family members, c-Rel, RelB, p65 (RelA), p105/p50 (NF-κB1) and p100/p52 (NF-κB2) that can form a range of homo- and heterodimers (9). After regulated IκB (inhibitor of NF-κB)-dependent NF-κB translocation to the nucleus, these dimers bind with variable affinities to consensus NF-κB-binding sites in the promoter and enhancer regions of their target genes, often cooperatively with other transcription factors [e.g. IFNβ promoter (10)]. This integrates other signal transduction pathways with the NF-κB pathway giving additional levels of specificity and regulation to the transcriptional control of responsive genes. The interaction with cell-type-specific co-factor proteins has been shown to influence the transcriptional potential of NF-κB (11). One of the co-factors of NF-κB is the co-activator p300 and its homolog CBP (CREB-binding protein). They have been shown to interact with the RelA/p65 and the p50 subunit serving as molecular bridges between NF-κB and the transcription machinery (8,10,12–14). They contain intrinsic histone acetyltransferase activity catalyzing the acetylation of lysine residues in histones and non-histone proteins (15,16). A growing number of transcription factors are acetylated and regulated by p300/CBP including p53 (17), GATA-1 (18), E2F-1 (19,20) and YY1 (21). Post-translational acetylation influences different properties of these transcription factors such as DNA binding, protein–protein interactions, protein stability and transcriptional potential (22).

NF-κB is subject to a variety of post-translational modifications [e.g. phosphorylation (23), ubiquitination (24) or prolyl-isomerisation (25)] that modulate

*To whom correspondence should be addressed. Tel: +41 44 6355474; Fax: 41 44 6356840; Email: hottiger@vetbio.uzh.ch

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

its activity. Phosphorylation of the RelA/p65 subunit by the PKAc, MSK1 and PKCζ kinases enhances its interaction with the co-activator p300/CBP and stimulates the NF-κB transcriptional activity (26–28). In contrast, ubiquitination of RelA/p65 on the promoter specifically terminates the NF-κB response (24).

It has recently been shown that RelA/p65 and p50 are reversibly acetylated by p300 and PCAF (29–31). Chen *et al*. identified lysine residues (K) 218, 221 and 310 of RelA/p65 as acceptor sites for p300 acetylation. They reported that lysine 221 acetylation enhanced DNA-binding activity of NF-κB *in vitro* and abolished the interaction with IκBα leading to a prolonged NF-κB response in the nucleus. The acetylation at lysine residue 310 was required for full transcriptional activity of RelA/p65 (32). Kiernan *et al*. identified lysine 122 and 123 in RelA/p65 as acetylation sites modified by both p300 and P/CAF. In contrast to K218, K221 and K310, acetylation of K122 and K123 decreased the DNA binding of RelA/p65 facilitating the removal of RelA/p65 from the DNA and the export from the nucleus by IκBα resulting in a faster termination of the NF-κB response (30). Furthermore, a recent report presented the TGF-β1 mediated acetylation of RelA/p65 at lysine 221 *in vitro* and *in vivo* enhancing the induced activation of NF-κB by bacteria (33).

Together, these data question the precise functional relevance of RelA/p65 post-translational acetylation in NF-κB-dependent gene regulation *in vivo*. Thus, our study aimed to identify the role of RelA/p65 acetylation *in vivo*. We found that p300 efficiently acetylated RelA/p65 *in vitro* and in cells at lysine 314 and 315—two novel acetyl acceptor sites. Additionally, our results confirmed the acetylation of RelA/p65 at the previously reported site of lysine 310 *in vitro* and *in vivo*. We generated acetylation-deficient lysine to arginine substitution mutants of RelA/p65 and stably complemented murine RelA/p65–/– cells with these mutants. The nuclear-cytosolic shuttling and the DNA binding of the acetylation-deficient mutants were similar to that of wild type RelA/p65. Furthermore, induction of anti-apoptotic genes by TNFα was not affected by the non-acetylatable mutants of RelA/p65. However, whole genome microarray analysis after TNFα stimulation indicated that the expression of specific genes was either positively or negatively affected by the K/R mutations. Our results imply that although general transcriptional activity of RelA/p65 was not affected by acetylation at lysine 310, 314 and 315, the expression of specific sets of genes was modulated by lysine-specific acetylation of RelA/p65. Thus, site-specific acetylation could serve as molecular mechanism to promote specificity of NF-κB-dependent gene expression.

MATERIAL AND METHODS

Plasmids

hGCN5L, mP/CAF and hTip60 were cloned into pFastBacHTb vector in frame with an N-terminal 6×His-tag. pph-CMV-Km-RelA/p65 wild type was previously described in (13). pph-CMV-Km-RelA/p65K310R (K310R), pph-CMV-Km-RelA/p65K314/K315R (K314/315R) and pph-CMV-Km-RelA/p65K310R/K314/K315R (KTR) were generated by site-directed mutagenesis according to the QuickChange protocol (Stratagene) using the following oligonucleotides:

K310R: 5′CGTAAAAGGACATACGAGACCTTCA**G**GAGCATCATGAAGAAGAGTCC3′,

5′GGACTCTTCTTCATGATGCTC**C**TGAAGGTCT CGTATGTCCTTTTACG3′,

K314/315R: 5′CCTTCAGGAGCATCATG**CG**GA**G**G AGTCCTTTCAGCGGACCC3′,

5′GGGTCCGCTGAAAGGACTC**C**TC**CG**CATGA TGCTCCTGAAGG3′ (bold letters represent K/R mutation). pphCMV-Km-RelA/p65K122/123R, pphCMV-Km-RelA/p65K218/221R, pphCMV-Km-RelA/p65K218/221/310R were generated using the QuickChange site-directed mutagenesis protocol with pph-CMV-Km-RelA/p65 wild type as template vector. The specific primer sequences can be received upon request. The combinatorial mutant pphCMV-Km-RelA/p65K218/221/310/314/315R (KQR) was generated with the same protocol using pph-CMV-Km-RelA/p65K310R/K314/K315R as template vector. All introduced mutations were confirmed by sequencing.

Reagents and antibodies

Mouse TNFα, Trichostatin A (TSA), Nicotinamide (NAM), acetyl-Coenzyme A, calf thymus core histones (H7755) and Trichloroacetic acid (TCA) were purchased from Sigma. Sodium fluoride (NaF) and beta-glycerophosphate were purchased from Flucka. ^{14}C-labeled acetyl Coenzyme A (MC269) was obtained from Moravek Biochemicals. Most of antibodies were from Santa Cruz Biotechnology: anti-RelA/p65 (C-20, sc-372), anti-α-tubulin (TU-02, sc-8035), control mouse IgG (sc-2025) and anti-PCNA (PC10, sc-56). The anti-p300 monoclonal antibody was purchased from BD Pharmingen (554215). The anti-p50 antibody was a generous gift from N. Rice (National Cancer Institute, Frederick, MD). Anti-myc 9E10 antibody was either purified from hybridoma cells according to standard protocol or purchased from Roche Applied Science. The polyclonal anti-acetylated-Lysine antibody was from Cell Signaling. A specific antibody against acetylated lysine 310 of RelA/p65 was generated in collaboration with Abcam. The anti-Ccl-7 antibody was purchased from Abcam (ab9911).

Tissue culture, cell transfections

Complemented RelA/p65–/– NIH 3T3 mouse embryonic fibroblasts (MEFs) and HEK 293T cells were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin/streptomycin and non-essential amino acids (GIBCO). Cells were transfected using the calcium phosphate precipitation method.

Generation and purification of baculovirus expressed proteins

All recombinant proteins were expressed in Sf21 cells using the Bac-To-Bac (GIBCO) or BacPAK (Clontech) system. Recombinant His-tagged proteins were purified over Ni^{2+}-beads (ProBond, Invitrogen).

In vitro acetylation assay

One microgram of recombinant human wild type or mutant RelA/p65 was incubated with 0.5–1 μg recombinant p300 or CBP or equimolar amounts of hGCN5L, mP/CAF or hTip60 in HAT buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 1 mM sodium butyrate) supplemented with 1.5 nmol ^{14}C-acetyl CoA for 45 min at 30°C. Reactions were stopped by adding 10 × Laemmli-buffer and proteins resolved on SDS–PAGE with subsequent visualization by Coomassie brilliant blue or SyproRuby staining. The gel was immersed in 1 M sodium salicylate for 20 min at RT. After drying, the gel was exposed to X-ray films (Contatyp) at –80°C.

MS/MS

In vitro acetylated RelA/p65 was resolved on SDS–PAGE, fixed and stained with Coomassie brilliant blue. The corresponding protein band was then excised and washed twice with 50% acetonitrile. After tryptic digestion the protein sequence analysis was performed at the Harvard Mass Spectrometry and Proteomics Resource Laboratory by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a ThermoFisher LCQ DECA XP quadrupole ion trap mass spectrometer.

GST-pull down experiments

GST, GST-RelA/p65wt and GST-RelA/p65KTR proteins were immobilized on glutathione beads (Amersham Pharmacia) and incubated with purified his-p300 in binding buffer (20 mM Hepes pH 7.5, 60 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin) for 2 h at 4°C rolling. Glutathione beads were washed with binding buffer. Proteins were boiled, resolved on SDS–PAGE and subjected to western blot analysis using anti-his antibody (Qiagen).

Acetylation assay in cells

Myc-tagged RelA/p65 wild type, RelA/p65 acetylation-deficient mutants or control empty vector were co-expressed with p300 in HEK 293T cells. After 15 h of transfection, cells were treated with HDAC inhibitors (HDACi: 2 μM TSA, 5 mM NAM) alone or in combination with TNFα (30 ng/ml) for 30 or 45 min, respectively. Whole cell extracts were prepared (50 mM Hepes pH 7.9, 420 mM NaCl, 0.5% NP-40, 1 mM PMSF, 0.5 mM DTT, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin). One milligram of whole cell extract and 2 μg of anti-myc antibody were used for subsequent immunoprecipitation. The immunocomplexes were analyzed by standard western blot analysis using anti-acetylated Lysine antibody. Membranes were reprobed with anti-myc antibody.

Lentiviral complementation of RelA/p65–/– MEFs

Virus production and transduction of RelA/p65–/– MEFs were performed as described in (34). Briefly, HEK 293T cells were transfected with 3.5 μg of the envelope plasmid, 6.5 μg of packaging plasmid, and 10 μg of pTV-myc-RelA/p65 wild type, RelA/p65 K/R mutants or the control pTV vector. After 24 h the viral supernatant was harvested and used to infect RelA/p65–/– MEFs. Thirty-six hours post infection cells were split into selective medium containing 2.5 μg/ml Blasticidin (Sigma). Expression of recombinant proteins in the complemented cells was screened by western blot analysis. Pools of cells were used for further analysis.

Electrophoretic mobility shift assay (EMSA)

Binding reactions were carried out in a total volume of 20 μl containing 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM PMSF, 0.25 μg poly dI-dC, 7% (v/v) Ficoll/glycerol, 0.25 pmol HIV-LTR oligonucleotide containing 2 κB binding sites labeled 5′ with ATPγ^{32}P and 7 μg of nuclear extract. The reaction was incubated for 20 min on ice and then resolved using a 5% polyacrylamide gel in 0.5 × TBE. The gel was run at 150 V for 3 h, dried and subjected to autoradiography. When supershifts were performed the binding reaction was pre-incubated with the indicated antibody for 20 min on ice before the labeled oligonucleotide was added.

Immunohistochemistry

Cells were plated at the density of 45 000 cells per chamber on poly-L-lysine (Sigma)-coated chamber slides (LAB-TEK) and incubated overnight at 37°C and 5% CO_2. Next day the cells were treated with 30 ng/ml of TNFα for the indicated time. The cells were fixed in 4% paraformaldehyde and then permeabilized with 0.2% Triton-X-100/PBS. After blocking for 1 h in 2% BSA/0.1% Triton-X-100/PBS slides were incubated with anti-RelA/p65 C-20 antibody (1:300 dilution) followed by anti-rabbit Cy3 antibody (1:250 dilution, Jackson Immunology). The samples were washed and Vectashield mounting solution (Vector laboratories) was applied to prevent bleaching. Cells were visualized using an Olympus T50 microscope.

Whole cell extract preparation and immunoprecipitation for RelA/p65-p300 interaction

The complemented cell lines were treated with TNFα (30 ng/ml) for 30 min. Whole cell extracts were prepared (25 mM Hepes pH 7.9, 300 mM KAc, 1% NP-40, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 50 mM NaF, 20 mM beta-glycerophosphate). One milligram of whole cell extract was incubated with 2 μg of anti-p300 antibody or control IgG antibody for 2.5 h at 4°C. The immunocomplexes were analyzed by standard western blot analysis using anti-RelA/p65, anti-p300 and anti-tubulin antibodies.

Nuclear extract preparation and immunoprecipitation for *in vivo* acetylation of K310

Nuclear extracts were prepared as previously described in (35). Two hundred micrograms of TNFα (30 ng/ml) and HDAC inhibitor [TSA (2 μM), NAM (5 mM)] treated

nuclear extracts of complemented cells were incubated with 2 μg of anti-RelA/p65 C-20 antibody for 2.5 h at 4°C rolling in binding buffer (20 mM Hepes pH 7.9, 80 mM NaCl, 2.5 mM $MgCl_2$, 0.05% NP-40, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin). After incubation with protein G-sepharose beads (Amersham Pharmacia) for another hour, the immunocomplexes were extensively washed in washing buffer (20 mM Hepes pH 7.9, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.05% NP-40, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin). Proteins were boiled, resolved on SDS–PAGE and analyzed by western blot using the anti-RelA/p65ac310 antibody. Membranes were reprobed with anti-RelA/p65 antibody.

Cell survival assay

The complemented cells (pTV empty vector control, RelA/p65wt, RelA/p65K310R, RelA/p65K314/315R and RelA/p65KTR) were plated in 12-well dishes at a density of 90 000 cells/ml. After 24 h of incubation, cells were starved overnight with 0% FCS containing medium and then treated with TNFα (30 ng/ml) or left untreated for additional 10 h. The surviving cells were counted using Trypan blue. The ratios of TNFα-treated and untreated cells were calculated for each cell line. Each condition was carried out in duplicate. A representative assay of three independent experiments is shown.

RNA preparation

Total RNA was isolated three independent times from TNFα-treated lysates from the different complemented RelA/p65–/– MEFs with the 'Total RNA isolation kit' (Agilent Technologies). RNA quality was assessed with the RNA 6000 Nano kit using the Bioanalyzer 2001 (Agilent Technologies). Purified RNAs were converted into double-stranded cDNA and transcribed into Cy3-/Cy5 (PerkinElmer/NEN Life Science)—labeled cRNA using the 'Low RNA Input Linear Amp Kit' (Agilent Technologies). cRNA from wild type RelA/p65 cells was Cy5-labeled while the RelA/p65K/R mutant cRNAs were Cy3-labeled. The purification of the labeled cRNAs was performed with the RNeasy kit (Qiagen). Dye incorporation was measured on the ND-1000 Spectrophotometer (NanoDrop Technologies)

Gene expression profiling

Gene expression profiling was performed in the Functional Genomics Center Zurich using the two-colour Agilent Microarray system (Agilent Technologies). 1 μg of fragmented Cy5-labeled wild type and 1 μg of Cy3-labeled mutant cRNA were each co-hybridized on the Whole Mouse genome 60mer-oligo array (G4122A, Agilent Technologies) according to manufacturers protocol. The microarray analysis was performed in triplicates. Slides were scanned using the Agilent DNA microarray scanner and the scans were quantified with the Agilent Feature Extraction software.

Data analysis

Data analysis was performed with GeneSpring software (Silicon Genetics). For the statistical comparisons of the RelA/p65 mutants and RelA/p65 wild type we only considered genes that were present in either the mutant or the wild type. We declared a gene as present in a comparison, if the hybridisation intensity was in all mutant replicates or in all wild type replicates above 200. Student's *t*-test was used to compute the significance of differential expression and 0.01 was used as significance threshold. For each list of significant genes, the Benjamini–Hochberg false discovery rate was computed and is reported in the results section. Furthermore, genes were filtered according to their fold change and only genes exceeding a fold change of 1.5 up or down are reported.

Quantitative real time RT-PCR

Total RNA from untreated or 45 min TNFα-treated cell lines (pTV, RelA/p65wt, RelA/p65K310R, RelA/p65K314/315R and RelA/p65KTR) was reverse transcribed using the high capacity cDNA Archive kit according to manufacturers protocol (ABI). Real-time PCR was performed using mouse-specific TaqMan probes (Gene expression assays, ABI) for Ccl-7, Ifi-44, Ccl-20 and Gpb-2. TaqMan probes for 18SrRNA and Rps6 were used to normalize for differences in RNA input. Rotor-Gene3000A (Corbett) was used to perform the real-time PCR reactions and the REST program was applied for analysis (36). The figures show the averaged results of three independent experiments.

TCA protein precipitation and Ccl-7 protein detection

Complemented cells (RelA/p65wt, RelA/p65K310R, RelA/p65K314/315R and RelA/p65KTR) were starved with 0% FCS containing medium for 1.5 h and then treated with TNFα (30 ng/ml) or left untreated. After 4 h of TNFα treatment, the medium was collected and 250 ng of recombinant PCNA was added as a control for the TCA protein precipitation. The medium was incubated overnight at 4°C with one-fourth volume of TCA (100% w/v) and centrifuged. Pellets were washed twice with acetone and lysed with lysis buffer (50 mM Hepes pH 7.5, 420 mM NaCl, 2 mM EDTA, 0.5% NP-40, 15% glycerol, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin). Forty micrograms of proteins were analyzed by standard western blot analysis using anti-Ccl-7 and anti-PCNA antibodies.

RESULTS

RelA/p65 is acetylated *in vitro* by p300 or CBP

Since RelA/p65 was reported to interact directly with several histone acetyltransferases (HAT), we investigated whether RelA/p65 could serve as a substrate in an *in vitro* acetylation assay. We compared the ability of different HATs to acetylate RelA/p65 *in vitro*. Full-length RelA/p65 was incubated with recombinant p300, CBP, GCN5L, P/CAF or Tip60 (all proteins expressed and purified from

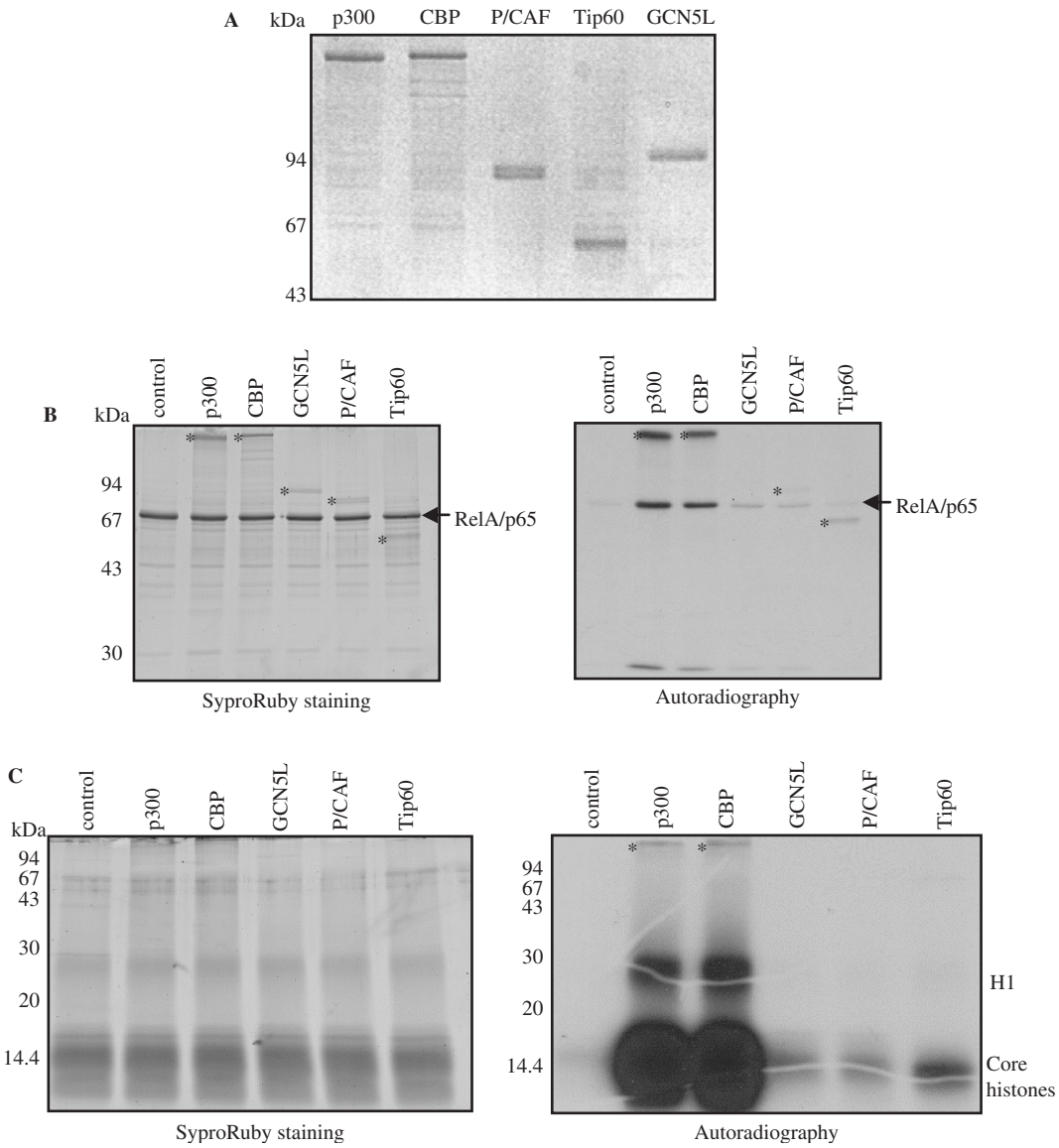


Figure 1. RelA/p65 is acetylated by p300 and CBP in *vitro*. (**A**) Recombinant histone acetyltransferases (HATs) expressed and purified from insect cells were analyzed by SDS–PAGE and Coomassie staining. Molecular weight markers are shown on the left. (**B**) *In vitro* acetylation assay using full-length RelA/p65 and indicated HATs. RelA/p65 was incubated in the presence of radioactively labeled [^{14}C]-acetyl-CoA with 500 ng of p300 or CBP or the equimolar amount of GCN5L, P/CAF or Tip60. Proteins were resolved on SDS–PAGE, stained with SyproRuby (left) and exposed to X-ray films (right). RelA/p65 acetylation signals are indicated with an arrow. HATs or HAT autoacetylation signals are indicated with an asterisk (*). (**C**) *In vitro* acetylation of histones. *In vitro* acetylation assay was performed as described in (**B**).

insect cells, Figure 1A) in the presence of radioactively labeled acetyl-coenzyme A (acetyl-CoA) as a donor of the acetyl group (Figure 1B). All tested HATs, except for Tip60, acetylated RelA/p65 *in vitro* (Figure 1B). Calf thymus core histones were used as a positive control of acetylation (Figure 1C). p300 and CBP were the most potent HATs for RelA/p65 in our system. This prompted us to focus on p300 in this study.

RelA/p65 is acetylated *in vitro* by p300 or CBP at lysine 310, 314 and 315

To identify the acetylation residues, *in vitro* acetylated RelA/p65 by p300 was digested with trypsin and the resulting peptides were analyzed by LC/MS/MS. 81.8% of the K310 comprising peptides contained an acetylated K310. 61.9% of K314 and 56.52% of K315 containing peptides showed acetylated K314 and acetylated K315, respectively. These data indicate that lysine 310, 314 and 315 were acetylated by p300. The identified lysine residues are located close to the C-terminus of the Rel homology domain (RHD) (Figure 2A). To confirm these findings, the corresponding lysines were replaced with arginine residues by site-directed mutagenesis. Substitution of lysine to arginine maintains the positive charge of the residue and may cause only minimal changes in the local environment of the protein. Wild type or mutated RelA/p65 harboring K310R, K314/315R or all three

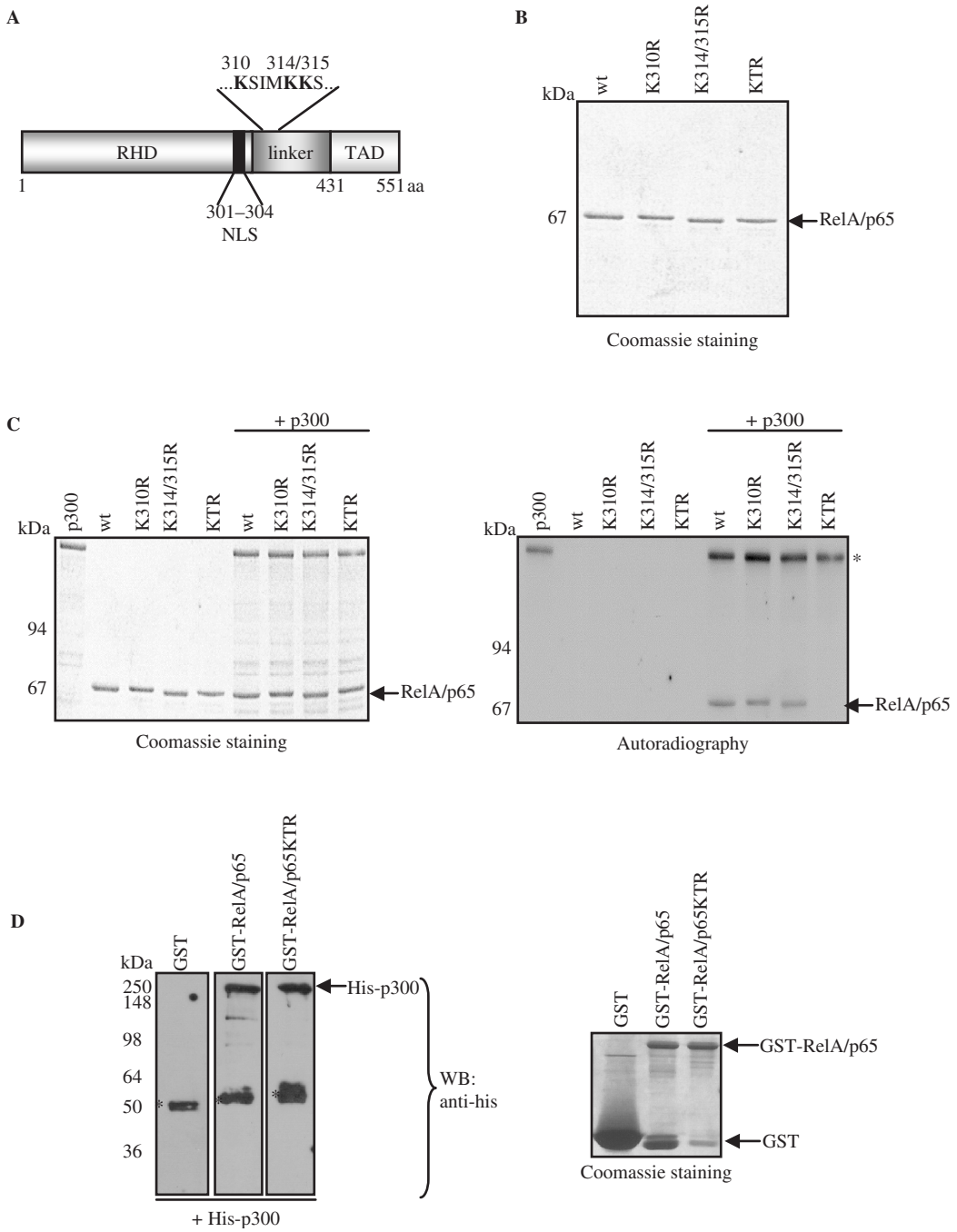


Figure 2. RelA/p65 is acetylated by p300 at lysine 310, 314 and 315 *in vitro*. (**A**) Protein domain diagram of RelA/p65 depicts the location of the acetylated lysine residues in the 'linker' region of RelA/p65 (NLS, nuclear localization signal; RHD, Rel homology domain; TAD, transactivation domain). (**B**) Coomassie staining of recombinant arginine to lysine substitution mutants of RelA/p65 expressed and purified from insect cells: wt, RelA/p65 wild type; K310R, RelA/p65K310R; K314/315R, RelA/p65K314/315R and KTR, RelA/p65K310/314/315R. (**C**) *In vitro* acetylation of RelA/p65K/R mutants by p300. Proteins were analyzed as in Figure 1B. The autoacetylation signals of p300 are indicated with an asterisk (*). (**D**) GST-pull down experiments using GST, GST-RelA/p65wt and GST-RelA/p65KTR as bait proteins were performed in the presence of purified his-p300. Co-precipitated proteins were analyzed by western blot using anti-his antibody (left panel: arrow indicates his-p300, asterix indicate unspecific binding). Coomassie staining of the bait proteins for equal loading is shown on the right (arrows indicate GST, GST-RelA/p65wt and GST-RelA/p65KTR).

K310/314/315R (KTR) substitutions were expressed and purified from insect cells (Figure 2B). Subsequently, all proteins were subjected to *in vitro* acetylation by p300 or CBP (Figure 2C and Supplementary Figure 1). Acetylation of RelA/p65 mutated at single K310 or K314/315 was only slightly reduced compared to wild type, while mutation of all three lysine residues abolished acetylation of RelA/p65 (Figure 2C). When the purified

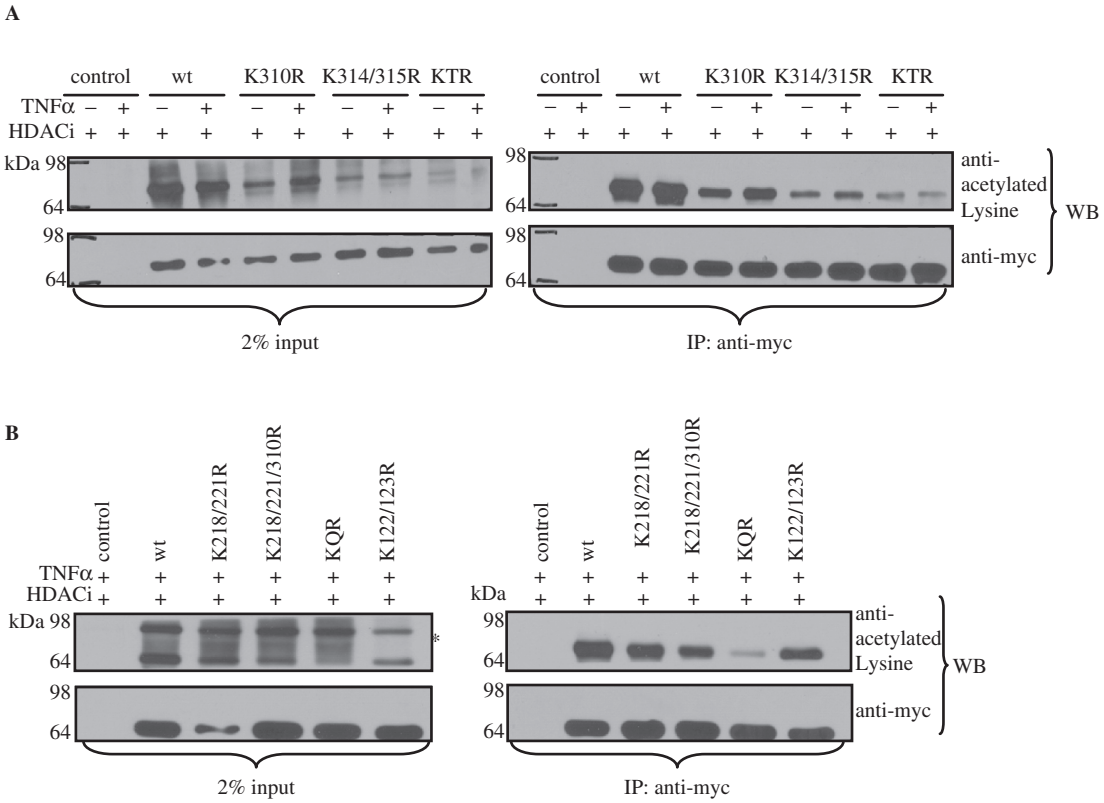


Figure 3. RelA/p65 is acetylated in cells on lysine 314 and 315 by p300. (**A**) Wild type myc-RelA/p65 and myc-RelA/p65K/R mutants were immunoprecipitated with anti-myc antibody from HDACi (TSA/NAM) and −/+ TNFα treated HEK 293T cells transfected with either empty control vector or different CMV-myc-RelA/p65 vectors (wt, K310R, K314/315R or KTR) along with CMV-p300. Western blot analysis of the immunocomplexes was performed after SDS–PAGE using anti-acetylated Lysine antibody (right panel). 2% input lanes are shown in the left panel. The membranes were reprobed with anti-myc antibody to assess equal input and immunoprecipitation. (**B**) HEK 293T cells were transfected with either control vector, myc-RelA/p65 wild type, myc-RelA/p65K122/123R, myc-RelA/p65K218/221R, myc-RelA/p65K218/221/310R or myc-RelA/p65KQR along with CMV-p300 and treated with HDACi (TSA and NAM) and TNFα. Wild type myc-RelA/p65 and myc-RelA/p65K/R mutants were immunoprecipitated with anti-myc antibody and the resulting immunocomplexes were resolved on SDS–PAGE. Western blot analysis was performed with anti-acetylated Lysine antibody. 2% input lanes are shown on the left. Membranes were reprobed with anti-myc antibody. Unspecific signals are indicted with an asterisk (*).

protein substrates were tested only in the presence of acetyl-CoA, no acetylation was observed confirming that the acetylation was mediated by p300 and not by a co-purified acetyltransferase. These results clearly indicate that K310, K314 and K315 of RelA/p65 are the main acetylation sites of p300 *in vitro*. To confirm that the RelA/p65KTR mutant was still able to interact with p300, GST-pull down experiments were performed using GST-RelA/p65 wild type or GST-RelA/p65KTR as bait proteins in the presence of purified p300. Subsequent western blot analysis revealed that p300 was able to equally directly interact with both RelA/p65 wild type and the RelA/p65KTR mutant (Figure 2D). These results indicate that abolished acetylation of the RelA/p65KTR mutant was due to the lack of specific sites and not due to the inability of this mutant to interact with p300.

Lysine 314 and 315 of RelA/p65 are acetylated by p300 in cells

To examine if lysine residues 314 and 315 of RelA/p65 are acetylated in cells, myc-tagged wild type RelA/p65, RelA/p65 acetylation-deficient mutants (RelA/p65K310R, RelA/p65K314/315R, RelA/p65KTR) or myc-tagged empty control vector were ectopically expressed in HEK 293T cells together with the histone acetyltransferase p300. Previous experiments had revealed that overexpressed RelA/p65 wild type can only be detected as acetylated in the presence of ectopically expressed p300 [(29) and data not shown]. TNFα was applied to induce NF-κB, while HDAC inhibitors (HDACi: TSA and NAM) were used to inhibit histone deacetylases. Subsequent immunoprecipitation analysis with anti-myc antibody and western blot analysis using anti-acetylated Lysine antibody revealed acetylation of RelA/p65 wild type (Figure 3A). The acetylation of wild type RelA/p65 was already close to saturation before TNFα stimulation most probably due to the activation of the NF-κB pathway through overexpression of RelA/p65. However, an increase of acetylation after TNFα stimulation is detected in the RelA/p65K310R mutant, strongly suggesting that lysine 314 and 315 are acetylated upon TNFα induction. A significant decrease in acetylation was detected in the RelA/p65 acetylation-deficient double

mutant (K314/315R). A small residual amount of acetylation was still detected when the triple mutant (KTR) of RelA/p65 was expressed in HEK 293T cells indicating that also other lysine residues are acetylated by p300 (Figure 3A).

To investigate if one of the previously reported sites [K122, K123 (30) and K218, K221 and K310 (32)] is acetylated in our system a variety of acetylation-deficient point mutants of RelA/p65 were generated: RelA/p65K122/123R, RelA/p65K218/221R, RelA/p65K218/221/310R and a combinatorial mutant RelA/p65K218/221/310/314/315R (RelA/p65KQR) harboring the acetylation acceptor sites identified in this study and the sites identified by Chen *et al*. after TNFα stimulation (32). The different mutants of RelA/p65 were expressed in HEK 293T cells along with p300 in the presence of TNFα and HDAC inhibitors. Anti-myc immunoprecipitation and anti-acetylated Lysine western blot analysis revealed a slight decrease in acetylation of the RelA/p65K122/123R and RelA/p65K218/221R mutant when compared to wild type RelA/p65 (Figure 3B). However, RelA/p65K218/221/310R showed a higher reduction in acetylation while the RelA/p65KQR mutant showed a decrease in acetylation of 90% (when compared to the wild type) indicating that K314 and K315 greatly contribute to the detected p300-dependent acetylation level in RelA/p65 after TNFα treatment. It also points out that K218 and K221 might probably be the other lysine residues acetylated in cells by p300 (Figure 3A). Together, these data indicate that RelA/p65 is acetylated in cells mainly on lysine 314 and 315 by p300.

Endogenous RelA/p65 is acetylated *in vivo* in TNFα stimulated cells

To investigate the role of RelA/p65 acetylation of K310, K314 and K315 *in vivo*, RelA/p65–/– NIH 3T3 mouse embryonic fibroblasts (MEF) were genetically complemented using lentiviruses encoding myc-RelA/p65 wild type, myc-RelA/p65K310R, myc-RelA/p65K314/315R or myc-RelA/p65KTR. After appropriate selection cells were kept in pools and the expression of recombinant proteins was analyzed by western blotting using an anti-RelA/p65 antibody (Figure 4A). The cells transduced with control virus encoding the resistance gene [mock infected (pTV)] and non-transduced wild type NIH 3T3 cells expressing endogenous RelA/p65 protein were included as controls. The expression levels of the recombinant wild type and mutated RelA/p65 proteins were comparable to that observed for endogenous RelA/p65 in NIH 3T3 cells. Furthermore, cell growth analysis revealed that the proliferation rate was comparable between the tested cell pools under normal growth conditions (data not shown).

We and others showed that RelA/p65 is acetylated in cells in a TNFα-dependent manner [Figure 3A and (29)]. In order to investigate whether endogenous RelA/p65 is acetylated at the identified lysine residues *in vivo*, antibodies directed against RelA/p65 peptides acetylated at residues 310, 314 or 315 were generated. Nuclear extracts were prepared after treating the complemented cells with TNFα and HDAC inhibitors (TSA and NAM). Upon TNFα treatment RelA/p65 nuclear import was induced in RelA/p65wt and RelA/p65 mutant complemented cells (Figure 4B). Immunoprecipitation with an anti-RelA/p65 antibody and subsequent western blot analysis using the specific acetyl K310 antibody revealed that RelA/p65 was indeed acetylated at this site upon TNFα treatment (Figure 4C, left panel). TNFα induced acetylation of RelA/p65 was further confirmed by immunoprecipitating RelA/p65 from untreated and TNFα stimulated cells (Figure 4C, right panel). Acetylated RelA/p65 could only be detected in the extracts stimulated by TNFα. Acetylation of RelA/p65 could not be detected in the input lanes due to low detection sensitivity of the anti-acetyl 310 antibody. Antibodies raised against acetylated 314 and acetylated 315 were found to be unspecific in this analysis (data not shown).

To investigate if the abolished acetylation of RelA/p65K310R and RelA/p65KTR was due to their inability to interact with endogenous histone acetyltransferase p300, immunoprecipitation analyses with the different mutants and p300 were performed. Whole cell extracts were prepared from wild type and mutant RelA/p65 complemented cell lines after TNFα stimulation to induce NF-κB. Immunoprecipitation with either anti-p300 or control IgG antibody and subsequent western blot analysis using anti-RelA/p65 antibody revealed that endogenous RelA/p65K10R, RelA/p65K314/315R and RelA/p65KTR are able to interact with endogenous p300 upon TNFα stimulation to the same extend as wild type RelA/p65 (Figure 4D).

K314/315R substitution mutation does not affect the shuttling, the DNA-binding ability or the protection against TNFα-induced cell death of RelA/p65

The complemented cells were treated with TNFα and subcellular localization of the recombinant RelA/p65 proteins was analyzed by immunofluorescence analysis at different time points (Figure 5A). Nuclear translocation of the recombinant RelA/p65 wild type was detected after 20 min of TNFα treatment and relocation to the cytoplasm after 60 min. A similar shuttling kinetics was observed for endogenous RelA/p65 protein in NIH 3T3 cells (data not shown). Analysis of the cells complemented either with K310R, K314/315R or KTR revealed no significant differences in the shuttling kinetics between mutated RelA/p65 and RelA/p65 wild type proteins (Figure 5A). This suggested that acetylation of RelA/p65 at the three tested lysine residues was not essential for the regulation of the RelA/p65 nuclear-cytoplasmic redistribution. This observation is supported by our findings that IκBα was able to bind to RelA/p65 wild type and the acetylation-deficient mutants with similar affinity (data not shown).

Next we investigated the influence of p300-mediated RelA/p65 acetylation on the ability of RelA/p65 to bind DNA. Nuclear extracts of the complemented cells treated with TNFα were tested in an electro mobility shift assay using an oligonucleotide containing two κB elements of the HIV-LTR promoter. The experiments revealed that

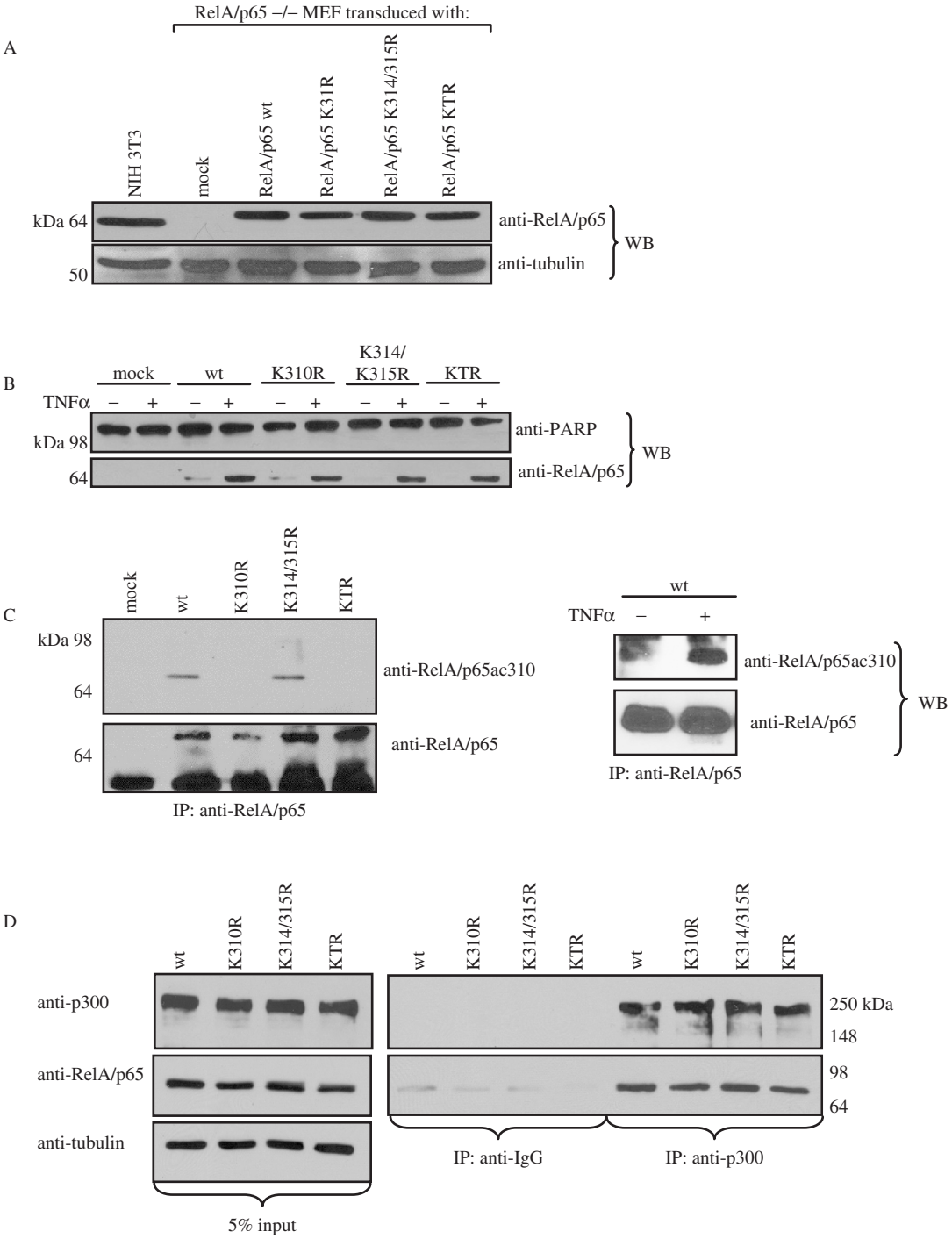


Figure 4. Endogenous RelA/p65 is acetylated at lysine 310 *in vivo* in response to TNFα. (**A**) Western blot analysis with anti-RelA/p65 antibody of whole cell extracts from RelA/p65–/– MEFs complemented with RelA/p65 wild type (wt) and the different substitution mutants of RelA/p65 (K310R, K314/315R and KTR). Mock transduced (pTV) and NIH 3T3 cells were used as controls. The membrane was reprobed with anti-tubulin antibody as loading control. (**B**) Nuclear extracts of the complemented cell lines untreated or treated with TNFα (30 ng/ml) for 30 min were subjected to western blot analysis using anti-RelA/p65 antibody. The anti-PARP western blot was performed to check extract fractionation. (**C**) In the left panel, nuclear extracts of the complemented cell lines treated with TNFα and HDACi (TSA/NAM) were subjected to immunoprecipitation analysis using anti-RelA/p65 antibodies. Western blot analysis with the anti-RelA/p65ac310 antibody was performed. The membranes were reprobed with anti-RelA/p65 antibody. In the right panel, RelA/p65 was immunoprecipitated from whole cell extracts of the complemented cell lines treated –/+ TNFα. The immunocomplexes were analyzed by western blot using anti-RelA/p65ac310 antibody and the membrane was reprobed with anti-RelA/p65 antibody. (**D**) Endogenous interaction analysis of RelA/p65 with p300 in TNFα-treated complemented cell lines. Endogenous p300 was immunoprecipitated from whole cell extract of complemented cell lines after TNFα stimulation for 30 min using anti-p300 antibody. Immunocomplexes were resolved on SDS-PAGE and subsequently analyzed by western blot using anti-RelA/p65, anti-p300 and anti-tubulin antibodies. Left panel: 5% inputs are shown, middle panel: IgG control immunoprecipitation; right panel: anti-p300 immunocomplexes.

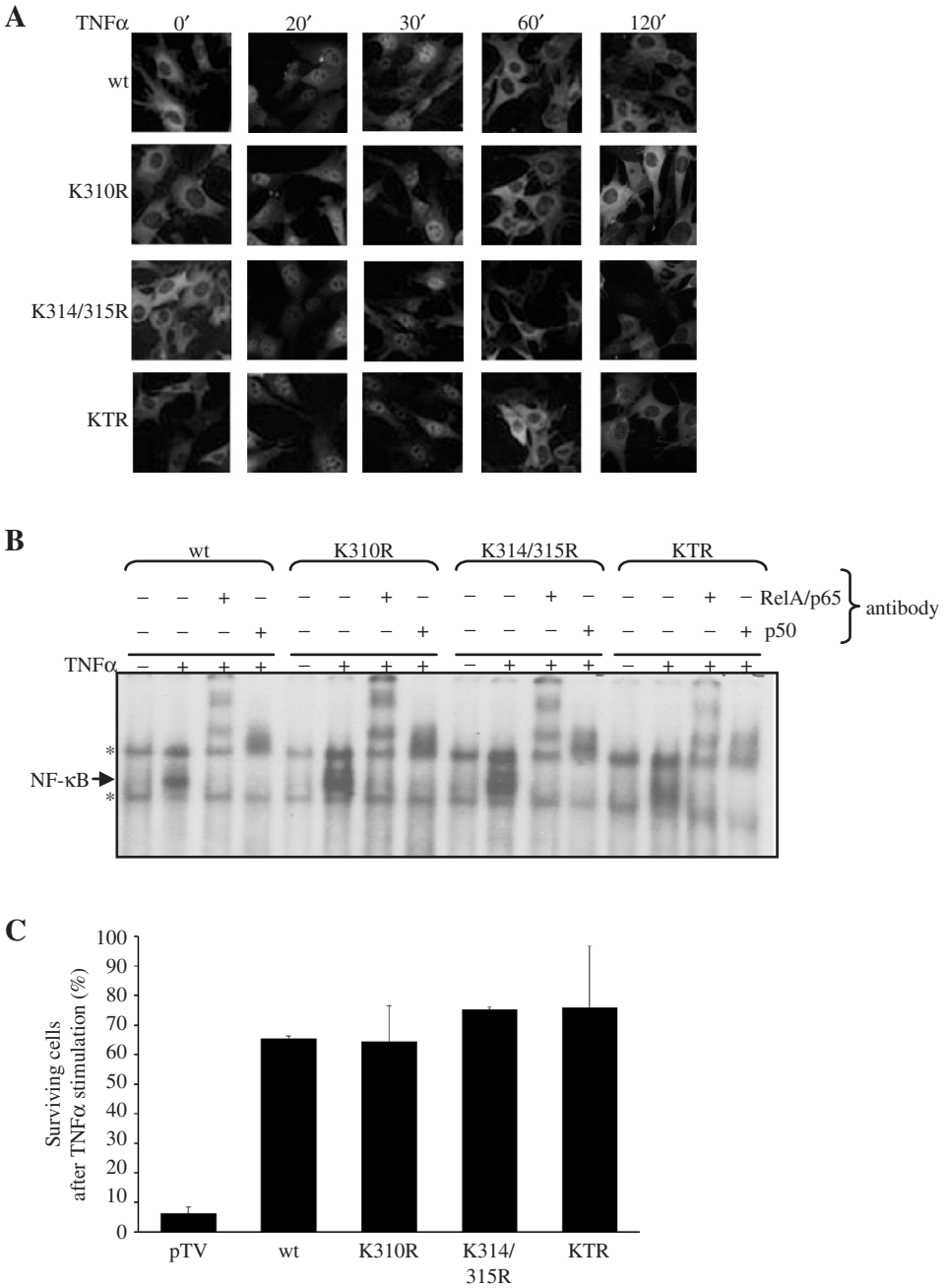


Figure 5. Mutation of the acetylation sites in RelA/p65 does not affect its nuclear translocation and DNA binding. (**A**) The complemented cells were treated with TNFα and fixed with paraformaldehyde followed by immunostaining using anti-RelA/p65 antibodies. Subcellular localization of RelA/p65 protein was analyzed by immunofluorescence microscopy. (**B**) Electro mobility shift assays were performed with nuclear extracts from the untreated or TNFα treated cells using ^{32}P-labeled oligonucleotide DNA containing 2κB sites. The bound complexes were characterized by anti-RelA/p65 or anti-p50 supershift assays. Specific NF-κB complexes are indicated with an arrow, unspecific bands are marked with an asterisk (*). (**C**) Cell viability assay after TNFα treatment. The different complemented cell lines (pTV, RelA/p65wt, K310R, K314/315R and KTR) were starved for 14 h with 0% FCS medium and then left untreated or treated with TNFα (30 ng/ml) for 10 h. The number of surviving cells (%) after TNFα treatment was calculated as described in 'Material and methods' section.

mutations of K310, K314 and K315 did not significantly influence the TNFα-induced DNA binding of RelA/p65 (Figure 5B). Binding was substantially reduced by competition with non-labeled oligonucleotides containing a wild type κB site, but not a mutated κB site, indicating that the binding was specific (data not shown). The presence of RelA/p65 and p50 in the complex was confirmed by supershift experiments using specific anti-RelA/p65 and anti-p50 antibodies, respectively (Figure 5B). Together, these findings show that the acetylation-deficient mutants are able to translocate to the nucleus and bind to DNA to the same extent as

RelA/p65 wild type. These results are in accordance with previously published data where K310 mutation to arginine did not affect subcellular localization, DNA binding or interaction with IκBα (32).

The deletion of the RelA/p65 gene in mouse cells is known to render them vulnerable to TNFα-induced cell death, stressing the important role of RelA/p65 in cell survival (37). To investigate whether TNFα-induced acetylation of RelA/p65 would affect the protective function of RelA/p65, cell viability assays were performed. The control cell line pTV (RelA/p65–/– cells complemented with empty vector), RelA/p65 wild type and the three different acetylation-deficient RelA/p65 mutant cell lines were either left untreated or were stimulated with TNFα for 10 h. The number of surviving cells after treatment was determined using Trypan blue and compared to the number of untreated cells (Figure 5C). As expected, the pTV cells were not protected from TNFα-induced cell death due to their lack of RelA/p65. However, the non-acetylatable mutants of RelA/p65 (K310R, K314/315R and KTR) protected the cells from cell death to the same extend as RelA/p65 wild type, indicating that the acetylation of RelA/p65 at the three mutated lysines does not affect substantially the tested pathway or that a possible effect could be compensated by the expression of RelA/p65-independent genes.

Determination of genes regulated by p300-mediated acetylation of RelA/p65 on lysine 314 and 315

To investigate the functional relevance of lysine 314 and 315 acetylation, microarray analyses were performed using the Agilent Whole Mouse Genome Array. The complemented cell lines (RelA/p65 wild type, RelA/p65K310R, RelA/p65K314/315R and RelA/p65KTR) were treated for 45 min with TNFα to induce NF-κB and total RNA was isolated in three independent replicates from these cells. RT-PCR for known TNFα-dependent NF-κB target genes was performed to check whether the TNFα induction was successful. The IκBα mRNA was induced to the same extend in the wild type and acetylation-deficient mutant cell lines (data not shown). RNA was amplified and labeled. The RNA from the different mutants (K310R, K314/315R and KTR) was then each co-hybridized with wild type RNA (RelA/p65 wild type cells) to the two-color whole mouse genome array. Statistical analysis of the TNFα-induced expression profiles aimed at identifying differentially regulated genes when comparing the different mutant cell lines with the wild type cell line. After performing this comparison, we identified following numbers of potentially regulated genes for K310R RelA/p65 versus wild type RelA/p65: 1149 significantly regulated genes with a P value ≤ 0.01 and a signal intensity > 200 at a false discovery rate of 18.61%. Thereof 79 genes were upregulated more than 1.5 times and 82 downregulated more then 1.5 times at a P value ≤ 0.01 (Supplementary Table S1). In the K314/315R RelA/p65 versus wild type RelA/p65 comparison we found 735 potentially regulated genes at $P \leq 0.01$ (signal intensity >200 and false discovery rate 28.43%). Ninety-seven genes were found to be upregulated with a fold change >1.5 and 54 downregulated with a fold change of <0.67 ($P \leq 0.01$) (Supplementary Table S2). When we finally analyzed the KTR mutant we found 1233 genes significantly regulated with a signal >200 and $P \leq 0.01$ at a false discovery rate of 17% (i.e. 216 false positives). Of these 1233 regulated genes 150 were upregulated with a fold change >1.5 and 116 downregulated with a fold change <0.67 ($P \leq 0.01$) when compared to wild type RelA/p65 (Supplementary Table S3). To investigate if common regulated genes were present in the different cell lines the overlap of the significantly downregulated and upregulated genes was generated at a P value ≤ 0.01 (Figures 6A and 7A). The overlap of genes between the different RelA/p65 mutants was small, possibly indicating distinct sets of genes being regulated by the different mutants.

To validate the results derived from the microarray studies, and to confirm the p65 dependency and TNFα induction of the analyzed genes, we performed quantitative RT-PCR for some selected genes. The following genes were chosen from the lists of overlapping differentially regulated genes at P value ≤ 0.01: Ccl-20, Ccl-7, Ifi-44 and Gbp-2. To perform quantitative real-time RT-PCR, RelA/p65–/– cells complemented either with the empty vector pTV, RelA/p65 wild type, RelA/p65K310R, RelA/p65K314/315R or RelA/p65KTR mutant were left untreated or were stimulated with TNFα for 45 min. Total RNA was isolated and reverse transcribed with random hexamer primers to obtain cDNA. Gene expression of Ccl-20, Ifi-44 and Gbp-2 was highly dependent on the acetylation of RelA/p65 at the identified sites, since only low levels of expression of these genes could be detected in cells expressing any of the mutants (Figure 6B–D, right panel, black bars). On the other hand, Ccl-7 expression was increased in cells expressing the K314/315R (2-fold) and the KTR mutant (2.59-fold) (Figure 7B, right panel). In the present analysis, the expression profiles obtained by microarray analysis were confirmed by quantitative RT-PCR analysis for all tested genes. However, the two methods showed higher correlation in the upregulated gene (Ccl-7; Figure 7B) than in the downregulated genes (Ccl-20, Ifi-44 and Gbp-2; Figure 6B–D).

In order to examine if these selected genes require RelA/p65 to be induced upon TNFα treatment, we compared their expression levels in the wild type cell line and in the control cell line pTV upon TNFα treatment. The four genes were upregulated in wild type cells, indicating that they were RelA/p65-dependent (left panels in Figures 6B–D and 7B). To determine the induction of these selected genes after TNFα stimulation in the different complemented cell lines, expression levels upon TNFα treatment were compared with unstimulated samples. All four genes were induced upon TNFα stimulation in wild type cells (middle panels from Figures 6B–D and 7B). However, among the downregulated genes, only Ccl-20 was stimulated after TNFα treatment in all acetylation-deficient mutants, though to a reduced amount than in the wild type cell line (Figure 6B–D). This suggests that gene expression of Ccl-20, Ifi-44 and Gbp-2 can only be properly induced by

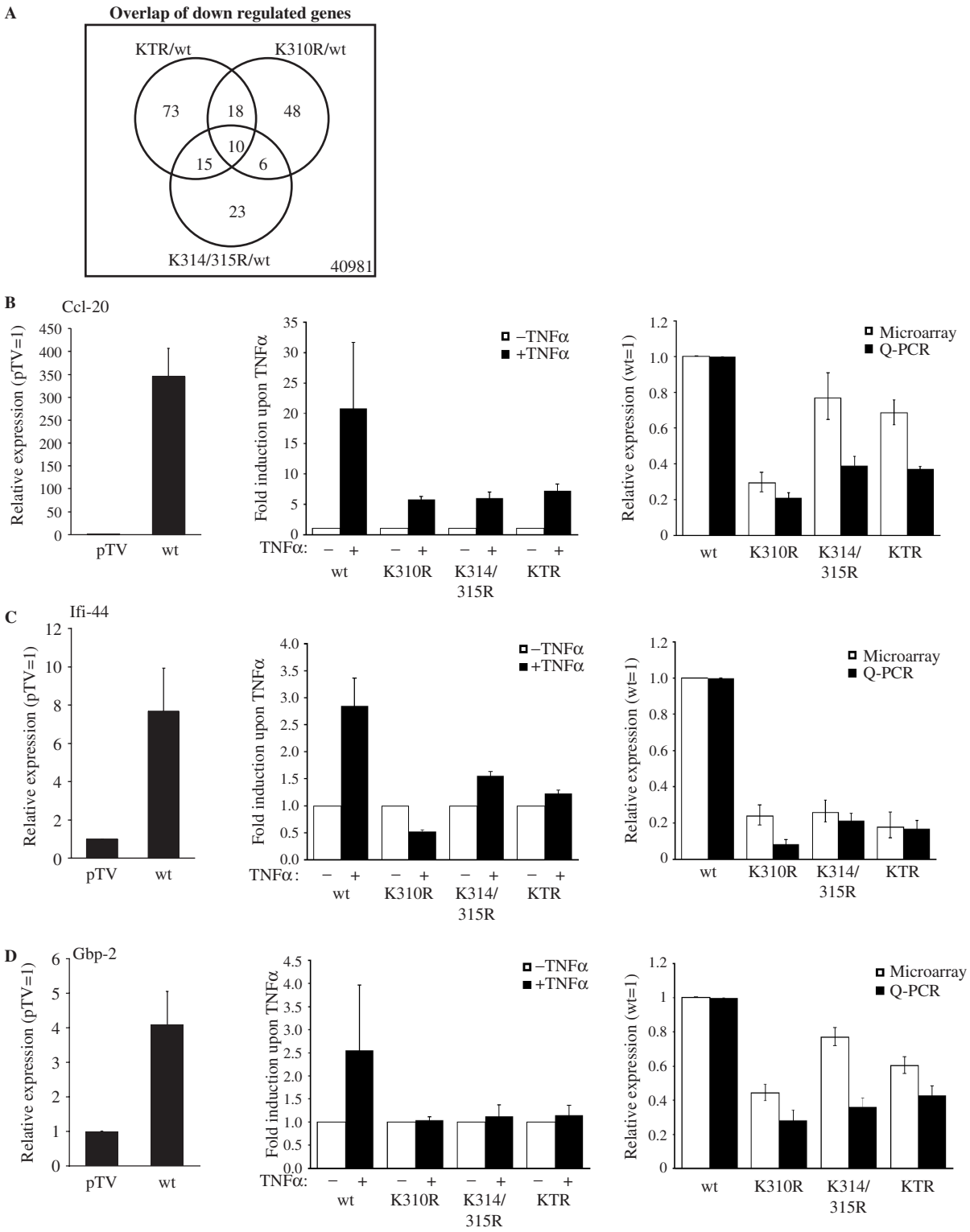


Figure 6. Acetylation of RelA/p65 at lysine 310, 314 and 315 activates gene specific transcription. (**A**) Venn diagram showing the numbers of common down regulated genes between the significantly regulated genes in the different RelA/p65 acetylation-deficient cell lines compared to the wild type cell line found in the microarray analysis. (**B**–**D**) Quantitative real time RT-PCR validation for selected downregulated genes, Ccl-20 (B), Ifi-44 (C) and Gbp-2 (D). Left panels show the comparison of relative expression in the presence of TNFα (pTV was set as 1). Middle panels show the TNFα fold induction of the different cell lines upon TNFα stimulation. Right panels show the comparison of relative expression of microarray (white bars) and real time PCR (black bars) experiments in the presence of TNFα (RelA/p65 wild type was set 1 in both methods). For real-time PCR 18SrRNA or Rps6 were used to correct for differences in cDNA inputs. The mean values from three independent, normalized measurements are shown with standard deviations.

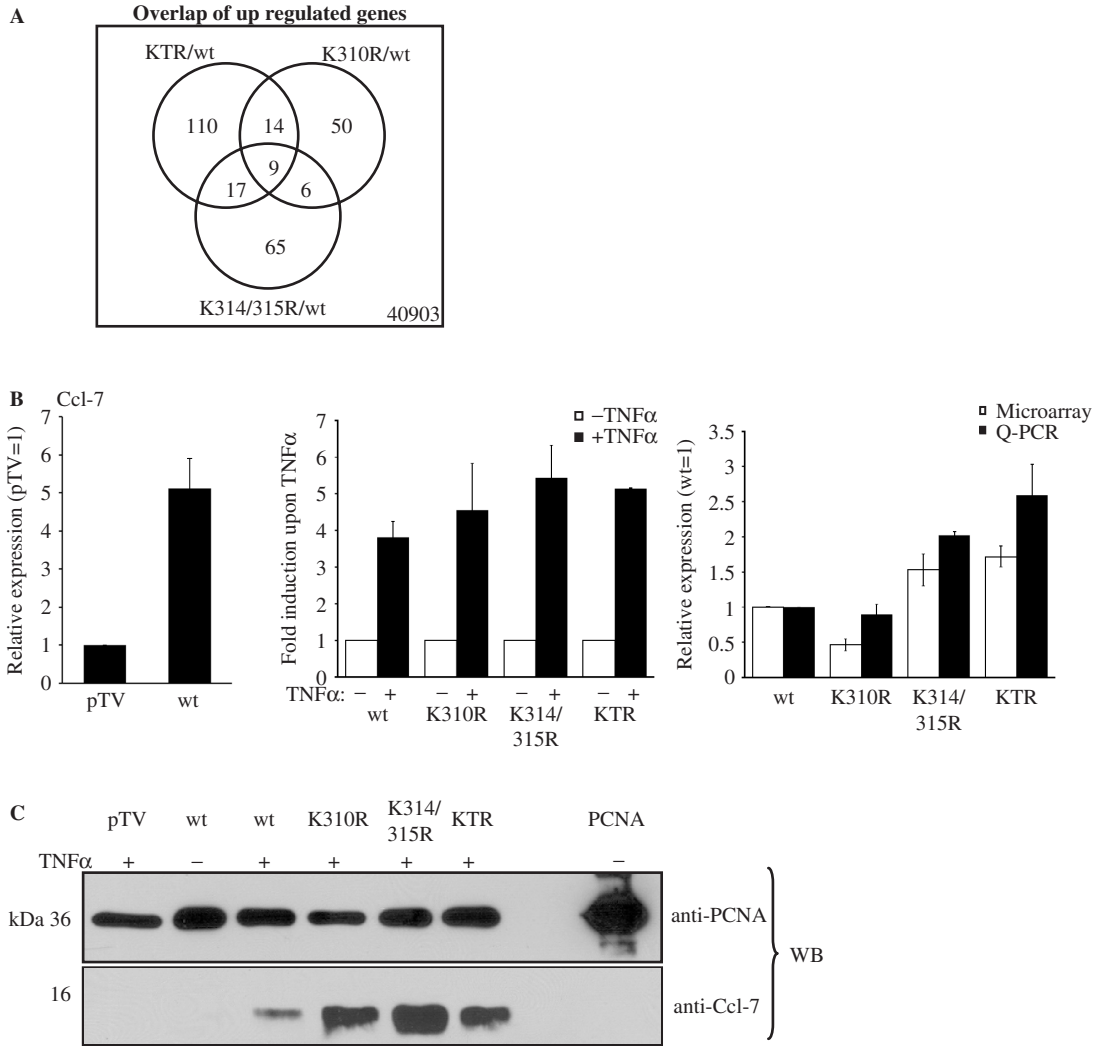


Figure 7. Acetylation of lysine 314 and 315 downregulates gene and protein expression. (**A**) Venn diagram of differentially upregulated genes found in the microarray analysis when the different RelA/p65 acetylation-deficient cell lines were compared to the wild type cell line. (**B**) Quantitative real-time RT-PCR validation for Ccl-7. The expression in the wild type cell line relative to pTV cell line in the presence of TNFα is shown in the left panel. The TNFα induction of this gene in the different cell lines is shown in the middle panel. The comparison between the microarray (white bars) and the real-time PCR (black bars) experiment is shown on the right side. The mean values from three independent, normalized measurements are shown with standard deviations. (**C**) Differential expression of Ccl-7 protein in acetylation-deficient cell lines of RelA/p65. Proteins present in the medium of complemented cells treated with TNFα for 4 h or left untreated were precipitated using TCA. Total 250 ng of recombinant PCNA was added as control to the collected medium before the precipitation was carried out. Western blot analysis was performed using anti-Ccl-7 and anti-PCNA antibodies. Total 250 ng of recombinant PCNA were loaded on the last lane of the SDS–PAGE to control the protein precipitation.

TNFα when RelA/p65 is acetylatable by harboring lysines at the position 310, 314 or 315. The Ccl-7 gene was induced by TNFα in all cell lines (Figure 7B, middle panel).

To demonstrate that transcriptional changes in gene expression correlate with changes in corresponding protein expression, western blot analysis was performed for Ccl-7 in the complemented cell lines. Upregulation of Ccl-7 protein was detected in the RelA/p65 acetylation-deficient mutant cell lines compared to wild type after 4 h of TNFα stimulation (Figure 7C).

Taken together, the results obtained in the microarray analysis could be confirmed by the complementary real time RT-PCR method for four selected genes. Interestingly, Ccl-7 protein expression analysis in the distinct acetylation-deficient cell lines indicated that the differential gene regulation detected in the microarray analysis is indeed translated into protein expression. Additionally, this analysis provides strong evidence that acetylation of RelA/p65 at lysines 310, 314 and 315 positively or negatively regulates the transcription of specific genes.

DISCUSSION

Increasing experimental evidence has indicated that NF-κB-dependent gene expression is regulated by different post-translational modifications including acetylation (38,39). The acetylation-dependent NF-κB regulation

was shown to occur via many different mechanisms (40). For example acetylation of histones is known to regulate NF-κB-dependent gene accessibility for the transcriptional machinery (38). The direct acetylation of NF-κB was reported to regulate the transcriptional potential of NF-κB, the duration of the NF-κB response, its DNA-binding activity as well as protein–protein interactions with several transcription cofactors (32,41,42).

In this study we have addressed the role of p300-mediated acetylation of RelA/p65 in the regulation of gene expression *in vivo*. We identified three lysines (K310, K314 and K315) in RelA/p65 as targets for the acetylation by p300 *in vitro* and in cells upon TNFα stimulation (Figures 2A and 3A). Interestingly, lysine 310 was previously shown to be acetylated by p300 (32). However, we additionally found two novel lysine residues, lysine 314 and 315, acetylated in RelA/p65 not reported before. It is important to mention that among the previously reported acetylation sites by Kiernan *et al.* and Chen *et al.* (30,32), we could only confirm the acetylation of K310 in our experimental system (Figure 3B). This could be due to the different experimental procedures; stimuli (TNFα versus PMA) and cell lines (HEK 293T cells versus Jurkat T cells) used in the studies and could point towards a very specific stimulus and cell-type-dependent regulation of RelA/p65 through acetylation. However, our results suggest additional yet to be identified p300 specific acetylation sites in RelA/p65 other than lysine 218, 221, 310, 314 and 315 (Figure 3C). The relevance of the *in vitro* acetylation sites identified in our study was confirmed by the ability of endogenous RelA/p65 to be acetylated *in vivo* upon TNFα stimulation using an acetylation site-specific antibody (Figure 4C).

To investigate the role of RelA/p65 acetylation in cells we genetically complemented RelA/p65–/– fibroblasts with a control vector (pTV) or cDNAs encoding for RelA/p65 wild type, K310R, K314/315R and KTR mutant (Figure 4A). Analysis of the complemented cells revealed that the mutation of the acetylation sites did not affect the kinetics of the cytoplasmic-nuclear redistribution of RelA/p65 upon TNFα stimulation (Figure 5A). This implied that the upstream signalling events are not regulated by the acetylation of RelA/p65 at the identified sites. Furthermore, cell survival assays were performed to examine whether the acetylation of RelA/p65 is involved in the protection from TNFα-induced cell death. No difference was seen in cell survival when RelA/p65–/– cells complemented with wild type RelA/p65 were compared to cells complemented with the acetylation-deficient mutants, indicating that the acetylation of RelA/p65 on lysine 314 and 315 is not involved in the TNFα-dependent cell death pathway (Figure 5C).

In order to elucidate the influence of RelA/p65 acetylation on gene expression *in vivo* we performed genome-wide microarray analyses using total RNA isolated from the complemented cells after TNFα stimulation. We identified subsets of genes, which were specifically modulated depending on the ability of RelA/p65 to be acetylated at lysine 310, 314 or 315 (Supplementary Tables 1–3). The number of common significantly down or upregulated genes found in the current analysis of the different cell lines is small, indicating that acetylation of RelA/p65 at lysine 310, 314 and 315 is possibly modulating the expression of only a subset of genes (Figures 6A and 7A). Four genes from the distinct subsets of differentially expressed genes were selected for validation: Ifi-44, Ccl-20 (Mip-3α), Gbp-2 and Ccl-7 (MCP-3); thereof Ccl-20 is a known NF-κB target gene (43). Ifi-44, Ccl-20 and Gbp-2 gene expression showed to be dependent on acetylation of RelA/p65 at lysine 310, 314 and 315. Expression of Ccl-7 was found to be repressed by acetylated RelA/p65. This was supported by the protein analysis performed for Ccl-7, which revealed induction of Ccl-7 protein expression in the acetylation-deficient mutant cell lines (Figure 7C). The increase in Ccl-7 protein only slightly correlated with the increase of mRNA levels detected in the different mutants at 45 min of TNFα stimulation (compare Figure 7B, right panel). This could be explained by the differences in stimulation duration for mRNA (45 min) and protein (4 h) detection and by additional regulatory mechanisms (such as secretion), which might influence the quantity of proteins.

When the induction of gene expression upon TNFα stimulation was investigated, differences were detected between the four genes. From the selected genes, the chemokines Ccl-20 and Ccl-7 were the only genes induced by TNFα in the mutant cell lines. Even though the upstream events of TNFα-induced NF-κB signalling were shown to be normal in our study, Ifi-44 and Gbp-2 gene expression was not induced by TNFα in the K/R mutant cell lines suggesting that regulation by K310, 314 and 315 acetylation is essential for the first group of genes (Ifi-44, Gbp-2) and required for the latter group (Ccl-20 and Ccl-7).

The results of the microarray analysis and the quantitative real-time PCR revealed that the acetylation of RelA/p65 does not always correlate with activation of gene expression as has been suggested before (32). Rather, we provide evidence that the p300-mediated acetylation of RelA/p65 contributes to both gene-specific activation and repression of transcription. It is unknown how RelA/p65 acetylation signals to activate certain genes while simultaneously suppressing the expression of others. One hypothesis would be that the regulation of certain genes by p300-mediated acetylation of RelA/p65 promotes the recruitment of additional factors or stabilizes the formation of specific pre-initiation complexes at promoter sites. Like this, acetylated RelA/p65 could serve as a factor that regulates the recruitment of these proteins. It has been previously reported that many proteins can specifically recognize and bind acetylated lysine residues in histones and transcription factors through their bromo domains (44–46). Among them are well-characterized histone acetyltransferases (e.g. p300, P/CAF and GCN5) (47), subunits of chromatin remodelling complexes (e.g. ATPases of SWI2/SNF2 and proteins of the WAL/BAZ family of ISWI-associated proteins) (48) and a general cofactor of the basal transcription machinery, TAF1 (49). The nature of the recruited co-activator/co-repressor would determine the response of the specific genes. Another hypothesis would be that the presence of other

cis-regulatory elements in a targeted gene and regulatory proteins recruited to these elements might be critical for the modulation of the acetylation dependent NF-κB response. Acetylation is known to be a reversible protein modification. In this regard HDAC-1, HDAC-2 and HDAC-3 were reported to repress NF-κB-dependent transcription upon treatment with inflammatory stimuli (32,38,40). These histone deacetylases were also shown to directly interact with several proteins involved in the NF-κB signalling pathway, including NF-κB itself (29,38,50). Deacetylation of RelA/p65 by HDAC-3 may provide a counterpart mechanism for p300 function. The exact molecular mechanism by which acetylation/deacetylation of RelA/p65 regulates the transcription activity of RelA/p65 in the context of chromatin remains to be investigated.

Together, acetylation of RelA/p65 presents an attractive regulatory mechanism for the control of NF-κB-dependent gene expression. The combination of acetylation with other post-translational modifications will broaden even more the potential of this regulation. Combinations of different modifications have already been proposed to serve as a 'code' for the interacting domains of different proteins (51,52). Furthermore, acetylation can be regulated by other post-translational modifications. For example phosphorylation of RelA/p65 at serine 276 and 536 was shown to enhance the acetylation of lysine 310 (53). It cannot be excluded that other post-translational modifications of RelA/p65 can also regulate the acetylation of this transcription factor. Our findings could help to explain the diversity of NF-κB-dependent gene expression upon different stimuli. We hypothesize that unique combinations of post-translational markers and the presence of cell-type-specific cofactors determine the specific NF-κB-mediated response. Recruitment analyses of RelA/p65 to the promoter region of the regulated genes using chromatin immunoprecipitation will help to understand how post-translational modifications of this transcription factor influence gene expression of its target genes.

In conclusion, our results support the hypothesis that p300-mediated acetylation of distinct sites in the RelA/p65 protein is important to modulate the expression of defined genes, thereby contributing to the specificity of the NF-κB response.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

Funding for this study was provided by Krebsliga Zürich; the Swiss National Science Foundation program (31-109315.05). T.V. and M.O.H. are supported by the Kanton of Zurich. We thank P.A. Cole (Johns Hopkins School of Medicine, Baltimore, MD), S. Dent (University of Texas MD Anderson Cancer Center), D. Trouche (LBME, University of Toulouse), Lee W. Kraus (Cornell University, Ithaca, NY), D. Thanos (BSRC Al. Fleming, Athens, Greece), E. Ferrari and U. Huebscher (IVBMB, University of Zurich) for providing useful tools. We are grateful to the Functional Genomics Center Zurich (FGCZ) for technical support (thanks especially to Andrea Patrignani for his assistance in data generation). We are also grateful to all the members of the Institute of Veterinary Biochemistry and Molecular Biology (University of Zurich, Switzerland) for helpful advice and discussions. Funding to pay the Open Access publication charges for this article was provided by Swiss National Science Foundation program 31-109315.05.

Conflict of interest statement. None declared.

REFERENCES

1. Bonizzi,G. and Karin,M. (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.*, **25**, 280–288.
2. Shishodia,S. and Aggarwal,B.B. (2002) Nuclear factor-kappaB activation: a question of life or death. *J. Biochem. Mol. Biol.*, **35**, 28–40.
3. Hayden,M.S. and Ghosh,S. (2004) Signaling to NF-kappaB. *Genes Dev.*, **18**, 2195–2224.
4. Pahl,H.L. (1999) Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*, **18**, 6853–6866.
5. Perkins,N.D. (2000) The Rel/NF-kappa B family: friend and foe. *Trends Biochem. Sci.*, **25**, 434–440.
6. Karin,M., Cao,Y., Greten,F.R. and Li,Z.W. (2002) NF-kappaB in cancer: from innocent bystander to major culprit. *Nat. Rev. Cancer*, **2**, 301–310.
7. Courtois,G. and Gilmore,T.D. (2006) Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene*, **25**, 6831–6843.
8. Perkins,N.D. (1997) Achieving transcriptional specificity with NF-kappa B. *Int. J. Biochem. Cell Biol.*, **29**, 1433–1448.
9. Karin,M. (1998) The NF-kappa B activation pathway: its regulation and role in inflammation and cell survival. *Cancer J. Sci. Am.*, **4 (Suppl. 1)**, S92–S99.
10. Thanos,D. and Maniatis,T. (1995) Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell*, **83**, 1091–1100.
11. Silkov,A., Wolstein,O., Shachar,I. and Dikstein,R. (2002) Enhanced apoptosis of B and T lymphocytes in TAFII105 dominant-negative transgenic mice is linked to nuclear factor-kappa B. *J. Biol. Chem.*, **277**, 17821–17829.
12. Gerritsen,M.E., Williams,A.J., Neish,A.S., Moore,S., Shi,Y. and Collins,T. (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl Acad. Sci. USA*, **94**, 2927–2932.
13. Hassa,P.O., Buerki,C., Lombardi,C., Imhof,R. and Hottiger,M.O. (2003) Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. *J. Biol. Chem.*, **278**, 45145–45153.
14. Perkins,N.D., Felzien,L.K., Betts,J.C., Leung,K., Beach,D.H. and Nabel,G.J. (1997) Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. *Science*, **275**, 523–527.
15. Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H. and Nakatani,Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87**, 953–959.
16. Bannister,A.J. and Kouzarides,T. (1996) The CBP co-activator is a histone acetyltransferase. *Nature*, **384**, 641–643.
17. Gu,W. and Roeder,R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595–606.
18. Boyes,J., Byfield,P., Nakatani,Y. and Ogryzko,V. (1998) Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature*, **396**, 594–598.
19. Martinez-Balbas,M.A., Bauer,U.M., Nielsen,S.J., Brehm,A. and Kouzarides,T. (2000) Regulation of E2F1 activity by acetylation. *Embo J.*, **19**, 662–671.

20. Marzio,G., Wagener,C., Gutierrez,M.I., Cartwright,P., Helin,K. and Giacca,M. (2000) E2F family members are differentially regulated by reversible acetylation. *J. Biol. Chem.*, **275**, 10887–10892.
21. Yao,Y.L., Yang,W.M. and Seto,E. (2001) Regulation of transcription factor YY1 by acetylation and deacetylation. *Mol. Cell. Biol.*, **21**, 5979–5991.
22. Glozak,M.A., Sengupta,N., Zhang,X. and Seto,E. (2005) Acetylation and deacetylation of non-histone proteins. *Gene*, **363**, 15–23.
23. Vermeulen,L., De Wilde,G., Notebaert,S., Vanden Berghe,W. and Haegeman,G. (2002) Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. *Biochem. Pharmacol.*, **64**, 963–970.
24. Saccani,S., Marazzi,I., Beg,A.A. and Natoli,G. (2004) Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor kappaB response. *J. Exp. Med.*, **200**, 107–113.
25. Ryo,A., Suizu,F., Yoshida,Y., Perrem,K., Liou,Y.C., Wulf,G., Rottapel,R., Yamaoka,S. and Lu,K.P. (2003) Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol. Cell.*, **12**, 1413–1426.
26. Zhong,H., Voll,R.E. and Ghosh,S. (1998) Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell.*, **1**, 661–671.
27. Vermeulen,L., De Wilde,G., Van Damme,P., Vanden Berghe,W. and Haegeman,G. (2003) Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *Embo J.*, **22**, 1313–1324.
28. Duran,A., Diaz-Meco,M.T. and Moscat,J. (2003) Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation. *Embo J.*, **22**, 3910–3918.
29. Chen,L., Fischle,W., Verdin,E. and Greene,W.C. (2001) Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science*, **293**, 1653–1657.
30. Kiernan,R., Bres,V., Ng,R.W., Coudart,M.P., El Messaoudi,S., Sardet,C., Jin,D.Y., Emiliani,S. and Benkirane,M. (2003) Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. *J. Biol. Chem.*, **278**, 2758–2766.
31. Furia,B., Deng,L., Wu,K., Baylor,S., Kehn,K., Li,H., Donnelly,R., Coleman,T. and Kashanchi,F. (2002) Enhancement of nuclear factor-kappa B acetylation by coactivator p300 and HIV-1 Tat proteins. *J. Biol. Chem.*, **277**, 4973–4980.
32. Chen,L.F., Mu,Y. and Greene,W.C. (2002) Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB. *Embo J.*, **21**, 6539–6548.
33. Ishinaga,H., Jono,H., Lim,J.H., Kweon,S.M., Xu,H., Ha,U.H., Xu,H., Koga,T., Yan,C., Feng,X.H. *et al.* (2007) TGF-beta induces p65 acetylation to enhance bacteria-induced NF-kappaB activation. *Embo J.*, **26**, 1150–1162.
34. El-Andaloussi,N., Valovka,T., Toueille,M., Steinacher,R., Focke,F., Gehrig,P., Covic,M., Hassa,P.O., Schar,P., Hubscher,U. *et al.* (2006) Arginine methylation regulates DNA polymerase beta. *Mol. Cell.*, **22**, 51–62.
35. Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
36. Pfaffl,M.W., Horgan,G.W. and Dempfle,L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.*, **30**, e36.
37. Beg,A.A. and Baltimore,D. (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*, **274**, 782–784.
38. Chen,L.F. and Greene,W.C. (2004) Shaping the nuclear action of NF-kappaB. *Nat. Rev. Mol. Cell Biol.*, **5**, 392–401.
39. Perkins,N.D. (2006) Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene*, **25**, 6717–6730.
40. Schmitz,M.L., Mattioli,I., Buss,H. and Kracht,M. (2004) NF-kappaB: a multifaceted transcription factor regulated at several levels. *Chembiochem*, **5**, 1348–1358.
41. Chen,L.F. and Greene,W.C. (2003) Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation. *J. Mol. Med.*, **81**, 549–557.
42. Deng,W.G., Zhu,Y. and Wu,K.K. (2003) Up-regulation of p300 binding and p50 acetylation in tumor necrosis factor-alpha-induced cyclooxygenase-2 promoter activation. *J. Biol. Chem.*, **278**, 4770–4777.
43. Tomimori,K., Uema,E., Teruya,H., Ishikawa,C., Okudaira,T., Senba,M., Yamamoto,K., Matsuyama,T., Kinjo,F., Fujita,J. *et al.* (2007) Helicobacter pylori induces CCL20 expression. *Infect. Immun.*, **75**, 5223–5232.
44. Dhalluin,C., Carlson,J.E., Zeng,L., He,C., Aggarwal,A.K. and Zhou,M.M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature*, **399**, 491–496.
45. Polesskaya,A., Naguibneva,I., Duquet,A., Bengal,E., Robin,P. and Harel-Bellan,A. (2001) Interaction between acetylated MyoD and the bromodomain of CBP and/or p300. *Mol. Cell. Biol.*, **21**, 5312–5320.
46. Mujtaba,S., He,Y., Zeng,L., Yan,S., Plotnikova,O., Sachchidanand, Sanchez,R., Zeleznik-Le,N.J., Ronai,Z. and Zhou,M.M. (2004) Structural mechanism of the bromodomain of the coactivator CBP in p53 transcriptional activation. *Mol. Cell.*, **13**, 251–263.
47. Yang,X.J. (2004) Lysine acetylation and the bromodomain: a new partnership for signaling. *Bioessays*, **26**, 1076–1087.
48. de la Cruz,X., Lois,S., Sanchez-Molina,S. and Martinez-Balbas,M.A. (2005) Do protein motifs read the histone code? *Bioessays*, **27**, 164–175.
49. Jacobson,R.H., Ladurner,A.G., King,D.S. and Tjian,R. (2000) Structure and function of a human TAFII250 double bromodomain module. *Science*, **288**, 1422–1425.
50. Ashburner,B.P., Westerheide,S.D. and Baldwin,A.S. Jr. (2001) The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol. Cell. Biol.*, **21**, 7065–7077.
51. Strahl,B.D. and Allis,C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41–45.
52. Jenuwein,T. and Allis,C.D. (2001) Translating the histone code. *Science*, **293**, 1074–1080.
53. Chen,L.F., Williams,S.A., Mu,Y., Nakano,H., Duerr,J.M., Buckbinder,L. and Greene,W.C. (2005) NF-kappaB RelA phosphorylation regulates RelA acetylation. *Mol. Cell. Biol.*, **25**, 7966–7975.

Deacetylation of p65 by cytoplasmic SIRT2 regulates NF-κB

Karin M. Rothgiesser[1], Susanne Waibel[2],

Bernhard Lüscher[2] and Michael O. Hottiger[1]*

[1]Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich

Winterthurerstrasse 190, 8057 Zurich, Switzerland

[2] Institut für Biochemie und Molekularbiologie, Medical School, RWTH Aachen

University, Pauwelsstrasse 30, 52074 Aachen, Germany

*Corresponding author:

Phone: +41-44-635 54 74, Fax: +41-44-635 68 40, E-mail: hottiger@vetbio.uzh.ch

Running title: SIRT2 is a NF-κB deacetylase

ABSTRACT (168 words)

NF-κB regulates the expression of a large number of target genes involved in the immune and inflammatory response, apoptosis, cell proliferation, differentiation and survival. In this study, we describe that acetylated p65, a subunit of NF-κB, is recruited upon TNFα stimulation to promoters of specific genes whose expression is regulated by the acetylation status of p65. In addition, we identified SIRT2 as a novel deacetylase of p65. SIRT2 is a member of the family of sirtuins, which are NAD^+-dependent deacetylases involved in several cellular processes. SIRT2 interacts with p65 in the cytoplasm and deacetylates p65 *in vitro* and *in vivo* at lysines 310, 314 and 315. Moreover, p65 is hyperacetylated at lysine 310 in *Sirt2*(-/-) cells after TNFα stimulation, which results in the dramatic increase in expression of a subset of p65 acetylation-dependent target genes. Together, our work provides strong evidence that p65 is deacetylated by SIRT2 in the cytoplasm after termination of the NF-κB response induced by TNFα, thus regulating the expression of specific NF-κB-dependent genes.

Keywords: NF-κB/ SIRT2/ acetylation/ p65/RelA

INTRODUCTION

Nuclear factor kappa B (NF-κB) belongs to a family of inducible transcription factors that modulates gene expression in response to a variety of extracellular and intracellular stimuli (4, 14, 29). NF-κB plays a crucial role in the regulation of many genes involved in mammalian immune and inflammatory response, such as cytokines, cell adhesion molecules, complement factors, and a variety of immune receptors. It has additionally been implicated as an important regulator of cellular processes such as apoptosis, cell proliferation and differentiation. The mammalian NF-κB family includes five members, p65 (RelA), c-Rel, RelB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2), encoded by *RELA*, *REL*, *RELB*, *NFKB1* and *NFKB2*, respectively (12). These proteins form homo- or heterodimers. The most abundant heterodimer in the majority of cells consists of the two subunits p65 and p50.

In most unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex through physical association with one of the inhibitors of NF-κB (IκB, predominantly IκBα). NF-κB induction involves the rapid activation of IKKβ and NEMO-dependent phosphorylation and subsequent degradation of IκBs. Consequently, dissociation of IκBs from NF-κB unmasks the nuclear localization sequence (NLS) of p65, which leads to nuclear translocation and binding of NF-κB to specific κB consensus sequences in the chromatin, to regulate specific subsets of genes (11, 26). Interestingly, one consequence of NF-κB activation is the upregulation of IκBα gene expression, mediated by a κB consensus sequence within the IκBα promoter (28). Several lines of evidence suggest that newly synthesized IκBα enters the nucleus, displaces non-chromatin associated subunits of NF-κB, thereby mediating their export from the nucleus and re-establishing a cytoplasmic pool of inhibited complexes (17). Relocation of NF-κB into the cytoplasm and attenuation of NF-κB-mediated transcriptional activation therefore provides a feedback mechanism for modulating the extent and duration of inflammatory responses by cells. Nevertheless, if cells are continuously exposed to a specific stimulus, NF-κB translocates back to the nucleus after some time of the first wave of NF-κB induction to continue with transcriptional regulation of target genes (15). The duration of NF-κB response depends in part on the stimulus and on the type of cell.

NF-κB specificity is regulated at different levels in the cell (30). It is plausible that different stimuli induce distinct posttranslational modifications of p65, determining the outcome of p65-mediated transcriptional events. Several posttranslational modifications on p65 have been described, the most important ones being phosphorylation and acetylation (29). Phosphorylation of p65 enhances its interaction with its transcriptional coactivator CBP/p300 and with components of the general transcription machinery (44). Acetylation of p65 was reported to modulate its DNA-binding capacity, its transcriptional activity, its interaction with IκB proteins and its subcellular localization (6, 20). We recently showed that p65 is acetylated by p300 not only at lysine 310, but also at lysine 314 and 315, two novel acetylation sites (5).

Microarray analysis of genetically complemented p65 knockout (-/-) cells treated with tumor necrosis factor α (TNFα) identified specific sets of genes differently regulated by acetylation deficient mutants of p65 compared to wild type cells. Together, these results showed that site-specific p300-mediated acetylation of p65 regulate the specificity of NF-κB-dependent gene expression.

Reversible protein acetylation is an important posttranslational modification that regulates the function of histones and many other proteins (34). Acetylation has a rapid turnover due to the highly dynamic equilibrium between two different groups of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). The balance between these two activities is key to regulate the appropriate cellular response to signals. Mammalian HDACs are divided into four main classes based on sequence similarity and cofactor dependency (Class I-IV) (42). Class III HDACs, also known as sirtuins, constitute a special class of enzymes because of their requirement for NAD^+ as a cofactor in their deacetylation reaction. During the deacetylation reaction, nicotinamide (Nam) is cleaved from NAD^+ and the acetyl group of the substrate is then transferred to ADP ribose, generating the novel metabolite 2'-O-acetyl-ADP ribose (OAADPr) (8). The sirtuin family consists of seven members in mammals (SIRT1–7) that contain a conserved catalytic core domain comprised of approximately 275 amino acids. They regulate a variety of cellular functions, such as genome maintenance, longevity, and metabolism (25, 39, 40). Up to date, all sirtuins have been described to have deacetylation activity except for SIRT4 (35). The SIRTs exhibit differential localization from nucleus to nucleolus, and from cytoplasm to mitochondria (33).

SIRT2 is the counterpart of yeast Hst2p, both proteins localize to the cytoplasm (31). SIRT2 was found to bind HDCA6 and to deacetylate α -tubulin, thereby participating in the regulation of microtubule dynamics and possibly cell cycle progression (9, 24, 27). Presumably, SIRT2 regulates other cellular functions, as p53, p300 and histones (H3 and H4) have been identified as substrates (3, 13, 37). The apparent contradiction of a protein being cytoplasmically located, yet exhibiting specificity for a histone residue was resolved when SIRT2 localization was monitored during cell cycle progression. SIRT2 localized to the cytoplasm throughout the cell cycle

with the exception of prophase during G2/M transition, where it translocated to the nucleus to deacetylate histone H4 at lysine 16 (H4K16) (37). Moreover, MEFs derived from *Sirt2*(-/-) mice showed hyperacetylation of H4K16 during mitosis.

Based on our recent findings, we extended our studies to further elucidate the molecular regulation of p65 acetylation. Here we identify additional genes regulated by p65 acetylation upon TNFα stimulation. Furthermore, we describe the characterization of SIRT2 as a novel deacetylase of p65. Gene expression analysis in SIRT2-deficient MEFs indicate that SIRT2-mediated p65 deacetylation is implicated in transcriptional regulation of a subset of genes.

RESULTS

Site-specific acetylation of p65 influences gene expression.

We provided earlier evidence that acetylation of p65 at lysines 310, 314 and 315 is important for the expression of a defined subset of genes (5). These earlier studies provided a first glance of the functional relevance of p65 acetylation, since gene expression was measured only after 45 minutes of TNFα stimulation. In order to know if the requirement for site-specific acetylation is maintained for the same genes after longer exposure to TNFα, and to identify possible new genes regulated through p65 acetylation, we decided to extent our analysis to 3 hours of stimulation. For this, we used *p65*(-/-) MEFs complemented with non-acetylatable mutants described previously, where the target lysines for acetylation were mutated to arginines (5). *p65*(-/-) cells complemented either with wild type p65, an empty plasmid as control (pTV), the acetylation-deficient double mutant K314/315R or the triple mutant KTR (K310/314/315R), were stimulated by TNFα for 3 hours and total RNA was isolated in three independent replicates from these cells. RNA was amplified, labeled and hybridized to the Agilent Whole Mouse Genome Array. After statistical analysis of the expression profiles, differentially expressed genes were identified (Fig. S1A-B, and Tables SI and SII). We focused only on genes that required p65 for their proper induction, which were identified by comparing

gene expression profiles from wild type and pTV cells. These experiments revealed that the majority of genes were upregulated in the double mutant K314/315R compared to wild type cells (Fig. S1A). In contrast, the majority of differentially expressed genes in KTR mutant were downregulated compared to wild type cells (Fig. S1B). The observation that mutation of lysines 314/315 increases expression of certain genes (e.g. *Mmp10* and *Mmp13*) suggests that either acetylation of these lysines represses gene expression or that acetylation would prevents modification of the same residues by another modification. Indeed, a recent study reported that these same two lysines (314/315) can by methylated by Set9 to induce ubiquitinylation of NF-κB and subsequently terminate gene expression (41).

Remarkably, only a few genes identified after 3 hours TNFα stimulation overlapped with the genes identified in our previous study after 45 minutes TNFα stimulation (data not shown), suggesting that acetylation is a very dynamic modification that regulates transcription accordingly.

We subsequently investigated the induction kinetics of several genes by real-time RT-PCR in the complemented cell lines stimulated by TNFα for different time points (between 20 and 360 minutes). Selection of these genes was based on their inducibility by TNFα, as well as their dependency on p65 and their regulation by acetylation of lysine 310 or 314/315. *Cfb, Mpa2l, NfkBie* represented genes downregulated in KTR, while *Mmp13* and *Mmp10* were upregulated in lysines 314/315 in the microarray experiments. Overall, the absolute mRNA levels of *Cfb, Mpa2l* and *NfkBie* were decreased whenever lysine 310 was substituted with arginine (Fig. 1A-B and Fig. S1C), while the ones from *Mmp10* and *Mmp13* were increased in the K314/315R mutant (Fig. 1C-D), corroborating our microarray results. Interestingly, some of these dependences were already observed when the basal expression levels were compared.

Chromatin-associated p65 is acetylated at lysines 310 and 314.

To further assess the functional relevance of p65 acetylation *in vivo,* we generated different antibodies raised against the acetylated lysines 310, 314 or 315. All raised

antibodies recognized their specific p300-mediated acetylated residues on recombinant p65 acetylated *in vitro* (Fig. 2A) or on overexpressed p65 acetylated *in vivo* (Fig 2B). These experiments revealed additionally, that acetylation of lysines 310 or 314/315 are not functionally interconnected, but that these residues are rather acetylated independently of each other.

From the above selected genes only *Cfb* was already described to contain a κB site in its promoter (18). We therefore searched for putative κB sites within the DNA sequence 1 kB upstream of the transcription start site (TSS) of the selected genes. This bioinformatic analysis identified several putative κB sites in the promoters of *Mmp10* and *Cfb*, one site in the promoter of *Mpa2l*, and none for *Mmp13* (Fig. S1D). *Mmp13* was thus not further investigated.

ChIP studies in *p65*(+/+) MEFs stimulated with TNFα for 20 and 180 minutes revealed that p65 was recruited to the promoters of *Cfb* and *Mpa2l* in a stimulus-dependent manner, while no enrichment was observed in *p65*(-/-) MEFs (Fig. 2C). These recruitments were promoter-specific, since p65 occupancy to promoter of *Glucagon*, a negative control, was not induced upon TNFα stimulation. *IP-10*, a known NF-κB target gene with very well characterized κB sites at its promoter, served additionally as a positive control. Unfortunately, no p65 enrichment could be observed to the κB sites of *Mmp10* (data not shown), indicating that p65 would activate this gene through other κB sites or other transcription factors.

Moreover, ChIP experiments using the anti-acetyl K310 and acetyl K314 antibodies showed that chromatin-associated p65 is indeed acetylated at lysines 310 and 314 (Fig. 2D). Unfortunately, the antibody raised against acetyl K315 was not able to immunoprecipitate p65 under the tested conditions (data not shown). Interestingly, recruitment of acetylated p65 lysine 310 or 314 to the promoter of *IP-10* was also observed. Together, these experiments identified *Mpa2l* as novel NF-κB target gene and provide strong evidence that p65 bound to chromatin is acetylated at lysines 310 and 314.

p65 is deacetylated by SIRT1 and SIRT2 at lysines 310, 314 and 315 *in vivo* and *in vitro*.

Since acetylation is a reversible posttranslational modification, we decided to investigate the kinetics of p65 acetylation in response to TNFα stimulation *in vivo*. *p65*(-/-) MEFs complemented with p65 wild type were stimulated for the indicated time points (20 to 90 minutes). Western blot analysis of immunoprecipitated p65 from the nuclear fraction of the cells revealed that endogenous p65 was rapidly acetylated at lysine 310 after 20 minutes of TNFα treatment (Fig. 3A). The signal dramatically decreased after 45 minutes of stimulation and was not detectable after 90 minutes, which correlates with the reported cytoplasmic relocalization of p65 in MEFs after TNFα stimulation (15). The sensitivity of the generated antibodies against acetyl K314 and acetyl K315 did unfortunately not allow the detection of endogenous p65 acetylated at those two lysines by western blot.

The reversed acetylation of p65 prompted us to investigate which enzyme is responsible for p65 deacetylation at the three lysine residues 310, 314 and 315. Initial experiments in HEK 293T cells overexpressing class I HDACs HDAC1, 2 and 3 did not reduce the acetylation levels of overexpressed p65 under the tested conditions (Fig. S2A). We therefore decided to focus on sirtuins. His-tagged SIRT1, 2, 6 and 7, known to localize either to the nucleus or to the cytoplasm, were overexpressed in HEK 293T cells (Fig. S2B), along with p65 and p300. Ectopic expression of p300 effectively led to p65 acetylation (Fig. 3B, mock sample). Notably, overexpression of SIRT1 and SIRT2 significantly reduced p65 acetylation at the three indicated lysine residues, while SIRT6 and SIRT7 were not able to deacetylate p65 under the tested conditions (Fig. 3B). To verify that the deacetylase activity of both SIRT1 and SIRT2 was necessary for p65 deacetylation, we overexpressed catalytically inactive SIRT1 and SIRT2 mutants (H363Y and H187Y, respectively), together with p65 and p300 in HEK 293T cells. Mutant SIRT1 and SIRT2 were inactive on the p65 substrate (Fig. 3C). These results indicate that the enzymatic activity of SIRT1 and SIRT2 are required to deacetylate p65 at lysines 310, 314 and 315 *in vivo*.

To confirm that SIRT1 or SIRT2 would directly deacetylate p65, recombinant p65 was acetylated *in vitro* by p300 and subsequently incubated with purified recombinant SIRT1, 2, 6 and 7 (Fig. S2C), in presence or absence of NAD^+. Both SIRT1 and SIRT2 were able to deacetylate p65 at lysine 310, 314 and 315 *in vitro*, as observed by western

blot analysis using the indicated antibodies (Fig. 3D). As expected, SIRT6 or SIRT7 were not able to deacetylate p65, in agreement with our previous results in HEK 293T cells (Fig. 3B).

Hyperacetylation of p65 at lysine 310 is observed in *Sirt2*(-/-), but not in *Sirt1*(-/-) cells.

SIRT1 has been previously reported to deacetylate p65 at lysine 310 (43). To further investigate the role of both SIRT1 and SIRT2 in p65 deacetylation *in vivo*, *Sirt1*(-/-) or *Sirt1*(+/+) and *Sirt2*(-/-) or *Sirt2*(+/+) MEFs were stimulated by TNFα and acetylation of endogenous p65 was analyzed by western blot. Only stimulation of *Sirt2*(-/-) cells by TNFα led to an increase in the acetylation of endogenous p65 at lysine 310 when compared with the other three cell lines (Fig. 3E). Protein levels of SIRT1 or SIRT2 were not altered in the analyzed MEFs when the other SIRT was knocked out (Fig. S2D). These results provide strong evidence that SIRT2 is the main enzyme responsible for p65 deacetylation at lysine 310 after TNFα stimulation *in vivo*.

SIRT2 interacts *in vivo* with p65 in the cytoplasm

To investigate whether endogenous SIRT2 and p65 would interact *in vivo*, SIRT2 was immunoprecipitated from cytoplasmic or nuclear extracts of human unstimulated Jurkat T-cells. Although the immunoprecipitation efficiency of SIRT2 was poor, SIRT2 could be detected in the cytoplamic but not in the nuclear extract. Interestingly, p65 coimmunoprecipitated with SIRT2 in the cytoplasmic extract (Fig. 4A).

TNFα stimulation does not induce nuclear translocation of SIRT2

Although SIRT2 has been reported to be located almost exclusively in the cytoplasm, we decided to investigate if it would translocate to the nucleus upon TNFα stimulation. Due to the low expression levels of endogenous SIRT2, we overexpressed HA-tagged SIRT2 in HeLa cells, followed by TNFα stimulation for 30 or 90 minutes. Consistent with our findings in Jurkat-T cells (Fig. 4A), immunofluorescence studies revealed that overexpressed SIRT2 and endogenous p65 localized to the cytoplasm in untreated cells

(Fig. 4B). Upon 30 minutes of TNFα stimulation, only p65 translocated to the nucleus and partly relocated to the cytoplasm after 90 minutes, while SIRT2 stayed in the cytoplasm throughout the experiment (Fig. 4B).

SIRT2 deacetylates p65 *in vivo* in the cytoplasm.

To provide further evidence for the cytoplasm as the cellular compartment where SIRT2 deacetylates p65, HEK 293T cells were transfected with expression plasmids for different SIRT2 mutants containing an additional NLS or an NLS in combination with a NES deletion (NLSΔNES). While wild type SIRT2 was located exclusively in the cytoplasm, fusion of an NLS to SIRT2 slightly increased its nuclear localization (Fig. 4C). Complete nuclear localization of SIRT2 could be observed only upon additional deletion of the NES (NLSΔNES mutant). Importantly, analysis of the p65 acetylation status under the same conditions revealed that the nuclear localization of SIRT2 negatively correlated with p65 deacetylation (Fig. 4D). The mutation of SIRT2 to achieve nuclear localization (SIRT2 NLSΔNES mutant) did not abolish its deacetylation activity, since it was able to efficiently deacetylate H4K16 in the nucleus (data not shown). Collectively, these results suggest that SIRT2 interacts with p65 exclusively in the cytoplasm for its deacetylation.

p65 deacetylation by SIRT2 contributes to regulation of NF-κB-dependent gene expression

Since p65 acetylation influences NF-κB-dependent transcription, we decided to investigate whether the expression of NF-κB target genes upon TNFα was affected by SIRT2 depletion *in vivo*. Gene expression analysis by real-time RT-PCR in *Sirt2*(+/+) and *Sirt2*(-/-) MEFs after 3 hours TNFα stimulation uncovered a dramatic increase in *Mpa2l* induction in *Sirt2*(-/-) cells compared to *Sirt2*(+/+) cells (around 70 fold difference) (Fig. 5A). This correlates with our previous results showing that acetylation of p65 at lysine 310 is required for transcriptional activation of *Mpa2l* and that p65 is hyperacetylated in *Sirt2*(-/-) MEFs. Since TNFα stimulation was continuous throughout the experiment, we expect p65 to have shuttled several times from the cytoplasm to the nucleus during those 3 hours, as also reported previously (15).

Next, we analyzed the expression of *Mmp13,* a gene with increased expression upon mutation of lysines 314/315. *Mmp13* induced gene expression was enhanced 3 fold in *Sirt2*(-/-) compared to *Sirt2*(+/+) cells (Fig. 5B). The induction of *RELA*, a gene that has neither been reported to be dependent on NF-κB nor to be a housekeeping gene (www.nf-kb.org), was not significantly different between the two genotypes (Fig. 5C). These data imply that the expression of genes that are particularly dependent on lysine 310 acetylation are strongly induced in *Sirt2*(-/-) cells, as observed for the strong *Mpa2l* expression. Together, these data suggest that deacetylation of p65 by SIRT2 influences NF-κB-mediated expression of *Mpa2l in vivo*.

DISCUSSION

We have previously shown that p65 acetylation plays an important role in regulating NF-κB-dependent transcription of a subset of genes. Our current results confirm those earlier findings by identifying new genes differentially regulated in the acetylation-deficient mutants compared to wild type cells. In addition, we report the identification of SIRT2 as a novel p65 deacetylase. SIRT2 deacetylates p65 *in vitro* and when overexpressed in cells after TNFα stimulation. Furthermore, endogenous p65 and SIRT2 interact in the cytoplasm of unstimulated cells. Subsequent TNFα treatment does not trigger SIRT2 translocation to the nucleus, thus indicating that it deacetylates p65 in the cytoplasm. Moreover, p65 is hyperacetylated at lysine 310 in *Sirt2*(-/-) MEFs, which correlates with a strong increase in *Mpa2l* expression, a gene which is dependent on the acetylation of lysine 310.

ChIP assays identified *Mpa2l* as a novel NF-κB target gene. *Cfb* was previously suggested to be a direct target of NF-κB by EMSA experiments (18). Here, we corroborated that p65 is recruited to this promoter in a TNFα-dependent manner *in vivo*. *Mmp10* and *Mmp13* were differentially expressed in acetylation-deficient mutant cells compared to wild type cells. However, we failed to detect p65 recruitment to the promoter of those genes. One explanation could be that p65 is recruited to the promoter region of these genes with a different kinetics than the one we investigated here.

Alternatively, p65 could bind to a regulatory element located far away from the promoter to regulate transcription, as has been shown for several NF-κB target genes (1, 10, 36). NF-κB is known to activate the expression of many transcription factors and their regulators (26); consequently, a third possibility is that p65 directly induces the expression of a protein that regulates the expression of these genes. Interestingly, NF-κB has been shown to directly activate the expression of the transcription factor Elk-1, which in turn induces *Mmp13* gene expression (23). A plausible explanation for our observations is that acetylated p65 at lysines 314 and/or 315 would help to control the expression of *Elk-1* in an acetylation-dependent manner, and thus expression of *Mmp13.*

The requirement of intact acetylation site at K310 in p65 for correct gene expression of *Cfb* and *Mpa2l* and the observation that acetylated p65 at lysines 310 and 314 is recruited to at least two of these promoters imply that p65 acetylation of K310 is required for transcriptional activation (Fig. 2D). The observed increase of acetylated p65 recruited to chromatin between 20 minutes and 3 hours of TNFα stimulation (Fig. 2D), in contrast to the reduction of p65 acetylated at lysine 310 in nuclear extracts already 45 minutes after stimulation (Fig. 3A), suggests that the pool of acetylated p65 bound to chromatin represents a minor fraction of the total p65 amount in the nucleus. Interestingly, our gene expression analysis indicated that the expression of *Cfb* and *Mpa2l* was not impaired in K314/315R mutant cells (Fig. 1A-B). Thus, it could well be that p65 is acetylated by p300 at the promoter of many genes, but that the expression of only some of these genes is affected by p65 acetylation at distinct lysines. This could also be the reason why we unexpectedly observed binding of acetylated p65 to the promoter of *IP-10*, a gene that did not show up in our microarray analysis to depend on p65 acetylation. Alternatively, p65 acetylation could be required for *IP-10* expression, although we did not detect this in the microarray analysis using *p65*(-/-) MEFs complemented with p65 wild type and non-acetylatable mutants. Indeed, we observed that TNFα does not induce NF-κB activity in the complemented MEFs as efficiently as in the parental *p65*(+/+) MEFs used for ChIP (data not shown). Therefore, although our complemented MEFs are useful to address the role of p65 acetylation *in vivo*, we might have identified only part of the genes influenced by p65 acetylation with the microarray screen.

Despite the identification of p65 acetylation, the exact mechanism by which acetylation of p65 regulates the expression remains to be further elucidated. By analogy with histone proteins, we can anticipate that the level of p65 acetylation reflects the competing influences of acetyltransferase(s) and deacetylase(s). An attractive possibility is that acetylated p65 creates docking sites for bromodomain-containing proteins that need to be recruited to promoters to modulate transcription. According to our findings, coactivators would bind to acetylated p65 at lysine 310. Recently, Huang *et al.* reported that double bromodomain of Brd4 binds to acetylated lysine 310, which enhances transcriptional activation of NF-κB and the expression of a subset of NF-κB inflammatory genes in an acetylated lysine 310-dependent manner (16). Future experiments will focus on the possible binding between acetylated p65 and bromodomain-containing proteins.

A recent study described methylation of p65 at lysines 314 and 315 and its involvement in the negative regulation of NF-κB-dependent transcription (41). Our ChIP experiments clearly show that chromatin-bound p65 is acetylated at lysine 310 as well as 314 in a promoter-specific manner. Based on the observation that chromatin-bound p65 is acetylated at lysine 310 as well as at lysine 314 at the same promoter, we reason that acetylation at lysine 314/315 could prevent methylation-mediated repression of target genes and thus positively influence transcription, together with acetylated lysine 310._A direct evidence for methylated p65 at lysines 314/315 bound to chromatin is still missing. Whether the same genes are regulated by both posttranslational modifications should be further addressed. Alternatively, both posttranslational modifications might regulate distinct set of NF-κB-dependent genes and thus not influence each other.

HDAC3 and SIRT1 have been previously described to deacetylate p65 (6, 43). However, we did not detect p65 deacetylation by overexpressed HDAC3 in HEK 293T cells (Fig. S2A). This disagreement in the results could be due to different experimental procedures, such as the use of HEK 293T cells instead of COS-7 cells. Deacetylation of lysine 310 by SIRT1 was shown under basal conditions in HEK 293T cells with overexpressed proteins and *in vitro* with recombinant proteins, which perfectly correlates with our results. SIRT1 and SIRT2 were both able to deacetylate p65 *in vitro*, but in

contrast to SIRT1 knock-out, only deficiency of SIRT2 was sufficient to induce hyperacetylation of p65 at lysine 310 upon TNFα stimulation *in vivo*. Our experiments with SIRT1(-/-)cells are in agreement with observations where RNAi of SIRT1 did not lead to changes in NF-κB target gene expression, unless cells were treated with resveratrol (43). These results suggest that SIRT1 and SIRT2 might function independently in p65 deacetylation and that they might regulate p65 in different cellular functions in response to different stimuli. Future experiments will address the role of p65 acetylation in gene expression for different cellular processes such as apoptosis, inflammation or immunity. Recently, a study showed that SIRT6 does not deacetylate p65 *in vitro* or *in vivo*, in agreement with our results (Fig. 3B and D), but deacetylates histone H3K9 to attenuate NF-κB signaling (19).

We observed that p65 was rapidly acetylated at lysine 310 in the nucleus of MEFs upon TNFα stimulation and that the signal was drastically reduced after 45 minutes of TNFα treatment (Fig. 3A). Immunostaining and EMSA experiments have previously shown that the majority of NF-κB shuttles back to the cytoplasm between 30 to 60 minutes after TNFα stimulation in MEFs (5, 15). Strikingly, the timing of p65 deacetylation at lysine 310 correlates with its reported export to the cytoplasm, supporting the idea that p65 is deacetylated in the cytoplasm.

Our data suggest that in unstimulated cells, SIRT2 exists in a complex with p65 and most probably p50 and IκBα, known to interact with p65 under basal conditions (Fig 6). Upon TNFα stimulation, the complex dissociates and p65/p50 heterodimers translocate to the nucleus, while SIRT2 stays in the cytoplasm. Nuclear p65 binds to specific κB sites in the promoter or enhancer regions of target genes together with transcriptional coactivators, such as p300. Consequently, p65 is acetylated at lysines 310 and 314/315 on the chromatin by p300, which influences its transcriptional activity, thus modulating the expression of a subset of target genes. Once the NF-κB response is terminated, p65/p50 heterodimer shuttles back to the cytoplasm, where SIRT2 deacetylates p65 at all three lysines, thereby resetting the whole NF-κB response. Any p65 that would afterwards translocate to the nucleus again during continuous TNFα stimulation would have to be freshly acetylated by p300 to modulate gene expression,

thereby allowing a tight control of NF-κB-dependent transcription.

Gene expression analysis of *Sirt2*(-/-) MEFs revealed that the transcriptional activation of *Mpa2l,* whose expression was dependent on p65 acetylation at lysine 310 (Fig. 1B), was dramatically increased in *Sirt2*(-/-) cells compared to other genes such as *Mmp13* or *RELA* (Fig. 5). Therefore, we propose that SIRT2 regulates the expression of a subset of NF-κB-dependent genes by deacetylating p65 in the cytoplasm upon relocation of NF-κB to this cellular compartment after the first wave of NF-κB induction. Since NF-κB is known to shuttle between nucleus and cytoplasm in 30-60 minutes periods in MEFs upon continuous TNFα stimulation (15), p65 would be hyperacetylated in *Sirt2*(-/-) cells after 3 hours of TNFα treatment, which would explain the observed differential *Mpa2l* gene regulation. Together, our results identify SIRT2 as a novel deacetylase of NF-κB and an important regulator of TNFα-induced NF-κB-dependent gene expression.

MATERIALS AND METHODS

Tissue culture

Complemented *p65*(-/-) NIH 3T3 mouse embryonic fibroblasts (MEFs) stably expressing p65 wild type or the acetylation-deficient mutants were previously described in (5). They, as well as the *p65*(-/-) and (+/+) parental NIH 3T3 MEFs kindly provided by A. Beg (2), were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin/streptomycin (Gibco) and non-essential amino acids (Gibco). *Sirt2*(-/-) and (+/+) MEFs were a generous gift from A. Vaquero. Those cells were originally generated in the lab of F. Alt (37). *Sirt1*(-/-) and (+/+) MEFs were kindly provided by F. Alt (7). *Sirt1*(-/-) , *Sirt1*(+/+), *Sirt2*(-/-) and *Sirt2*(+/+) MEFs were kept in DMEM supplemented with 15% FCS, 100 units/ml penicillin/streptomycin (Gibco), non-essential amino acids (Gibco), β-mercaptoethanol (Gibco) and Na-Pyruvate (Gibco). HEK 293T and HeLa cells were maintained in DMEM supplemented with 10% FCS and 100 units/ml penicillin/streptomycin; Jurkat T-cells in RPMI with 10% FCS and 100 units/ml penicillin/streptomycin.

Plasmids

Plasmids for the mammalian expression of human p65 wild type and mutants K310R, K314/315R and KTR were described elsewhere (5). Mammalian expression plasmids of human SIRT1, 2, 6 and 7 in the pcDNA-DEST40-V5/HIS background were kindly provided by I. Horikama (22). The enzymatic inactive mutant was generated by site-directed mutagenesis of H187 to Y with pcDNA-DEST40-SIRT2 as template. The introduced mutation was verified by DNA sequencing. pcDNA3.1-SIRT1-MYC/HIS

wild type and the catalytically inactive mutant (H363Y) were a generous gift from T. Kouzarides (21). Plasmids for the expression of HA-tagged SIRT2 and HA vector control were described elsewhere (27). The GW-pHA-SIRT2-NLS vector was created by cloning the sequence 5'-GATCCCCAAAGAAGAAGCGAAAGGTAC into GW-pHA-SIRT2. Site-directed mutagenesis was performed to change the SIRT2 lysines K4 and K12 to alanines, creating the GW-pHA-SIRT2-NLSΔNES vector. The introduced sequence and mutations were verified by DNA sequencing.

Reagents and antibodies

Human tumor necrosis alpha (TNFα), Trichostatin A (TSA), Nicotinamide (Nam) and acetyl-Coenzyme A (acetyl Co-A) were purchased from Sigma. Recombinant mouse TNFα was either purchased from Sigma or generated in our laboratory. Sodium fluorid (NaF) and beta-glycerophosphate were obtained from Fluka. The acetyl-specific antibodies for p65 anti-acetyl K310 (ab19870), anti-acetyl K314 (ab18727) and anti-acetyl K315 (ab19869) were generated by Abcam. The following antibodies were purchased from Santa Cruz Biotechnologies: anti-p65 (sc-372), anti-p300 (sc-585) and anti-SIRT2 (sc-20966 and sc-28298). The anti-SIRT1 antibody was from Millipore (07-131). The anti-myc antibodies were either purchased from Roche (11-667-149-001) or purified from hybridoma cells. The anti-tubulin antibody was purchased from Sigma (T 6199), the anti-his was from Qiagen (34670) and the anti-HA was from Covance (MMS-101P).

Generation of recombinant proteins

The recombinant proteins were expressed by baculovirus in Sf21 cells using either the FastBac or the BacPAK systems (Clontech). His-tagged proteins were purified over Ni^{2+}-beads (ProBond, Invitrogen) and GST-tagged proteins over L-Glutathione beads (Sigma). The virus for expression of GST-SIRT1 was kindly provided by K. Chua.

Microarray

Complemented cell lines p65 wild type or acetylation-deficient mutants were starved overnight before either left untreated or stimulated with 30 ng/ml TNFα for 3 hours. Total RNA from three biological replicates per sample was isolated at different days using the 'Total RNA isolation mini kit' (Agilent Technologies). RNA quality was measured on the 2100 Bioanalyzer (Agilent Technologies). Microarray experiments were performed using 'Whole Mouse Genome (4x44K) Oligo Microarray Kit' (Agilent Technologies) and 'One-color microarray-based gene expression analysis' (Agilent Technologies) following the manufacturer's protocol. Cy3-labeled cRNA was purified with the RNeasy kit (Qiagen). Dye incorporation was assessed with the ND-1000 Spectrophotometer (NanoDrop Technologies). Per sample, 1.65 μg cRNA from each of the three biological replicates was hybridized to independent arrays according to the manufacturer's protocol. Hybridized slides were scanned with the Agilent DNA Microarray scanner and quantified using the Agilent Feature Extraction software. The data analysis was performed using 'Rosetta Resolver® Gene Expression Data Management and Analysis System' (Rosetta Biosoftware). Briefly, data was processed and normalized with default settings. Then, low-signal genes with signal intensities <0.1

were filtered out. Differential expression between two conditions was assessed based on the average ratio and significance. All genes with expression ratios <0.556 and >1.8, and a p-value <0.05 were selected to generate the tables of significantly regulated genes. These sequence data have been submitted to the GEO database (www.ncbi.nlm.nih.gov/geo) under accession number GSE15196.

Gene expression by real-time RT-PCR

Complemented MEFs were starved overnight before treatment with 30 ng/ml recombinant TNFα for different time points. *Sirt2*(+/+) and *Sirt2*(-/-) MEFs were starved for 6 hours and then stimulated with 30 ng/ml recombinant TNFα for 3 hours or left unstimulated. Total RNA was isolated from at least two biological samples at different days with the 'Total RNA isolation mini kit' (Agilent Technologies). RNA was subsequently retro-transcribed using the 'High-capacity cDNA reverse transcription kit' (Applied Biosystems). Real-time PCR was performed using the Rotor-Gene 3000 (Corbett Life Science, now Qiagen) and TaqMan assays from Applied Biosystems for *Cfb, Mpa2l, Mmp10, Mmp13, NfkBie, Rps6* and *CanX* genes. The last two genes were used as internal controls to normalize for RNA input. In addition, gene expression of *Mpa2l, Mmp13, RELA* and *Rps12* (as internal control) was assessed with SYBR Green using the following primers: Mpa2l_forward (GATGCTGAAGAAGCTAATGAAGG ATC), Mpa2l_reverse (CCTTGATGACATCTCTCAGTTGCTG), Mmp13_forward (GGACAAGCAGTTCCAAAGGCTACA) and Mmp13_reverse (GTCTTCATCGCCTG GACCATAAAG). RNA from at least two biological replicates per sample was measured and analyzed with REST (32). Each experiment was run three independent times, the mean value and ± SD was calculated and blotted into graphs with GraphPad Prism (GraphPad Software).

Chromatin Immunoprecipitation

p65(-/-) or (+/+) MEFs were stimulated with 10 ng/ml mouse TNFα for the indicated time points and fixed with 1% formaldehyde (Calbiochem) for 10 minutes. After extensive washing, the plasma membrane was first lysed with lysis buffer 1 (50mM Tris HCl pH8, 2mM EDTA pH8, 0.1% NP-40, 10% glycerol, 1 mM PMSF, 0.5 mM DTT, phosphatase and HDAC inhibitors) and then the nuclear membrane with lysis buffer 2 (50mM Tris HCl pH8, 5 mM EDTA pH8, 1% SDS, 1 mM PMSF, 0.5 mM DTT, phosphatase and HDAC inhibitors). Chromatin fragmentation was achieved with the Bioruptor (Diagenode). Sonified chromatin was diluted with 9 volumes of dilution buffer (50mM Tris HCl pH8, 5mM EDTA pH8, 0.5% NP-40, 200mM NaCl and 1mM PMSF) and pre-cleared for 1 hour with Protein A Agarose/salmon sperm DNA (Millipore). 1% of input was saved and the remaining chromatin was then incubated overnight with the specific antibodies. After 30 additional minutes of incubation with Protein A Agarose/salmon sperm DNA, the immuno-complexes were extensively washed with washing buffer (20mM Tris HCl pH8, 2mM EDTA pH8, 1% NP-40, 0.1% SDS, 500mM NaCl and 1mM PMSF) and then with buffer TE (10mM Tris HCl pH8 and 1mM EDTA pH8). Chromatin was eluted with 2% SDS in TE buffer and incubated at 65°C for at least 6 hours. DNA was purified with 'QIAquick PCR purification kit' (Qiagen) following the manufacturer's recommendations and measured by real-time PCR using SYBR Green

and the Rotor-Gene 3000 (Corbett Life Science, now Qiagen). The following primers were used: Mpa2l_forward (CAGCCCCTTTTATAGTGAGTC), Mpa2l_reverse (TAC AAAATCCGGGAGTATTGC), Cfb_forward (CACCTGTGAAGCAAGTCTCTCTCT), Cfb_reverse (TTTGTGCAGCAAGGACTCTGACCT), IP-10_forward (GCAATGCCCT CGGTTTACAG), IP-10_reverse (GGCTGACTTTGGAGATGACTCA), Glucagon_ forward (GAGTGGGCGAGTGAAATCAT) and Glucagon_reverse (TGAGCTGCGA ACAGGTGTAG). Samples were normalized to input chromatin and expressed as % input. Each experiment was independently repeated at least three times. Mean values ± SD of three independent real-time PCR runs from one independent ChIP are shown.

***In vitro* acetylation assay**

1 μg of recombinant p65 wild type or the acetylation-deficient mutants were incubated with 500 ng recombinant p300 in HAT buffer (50 mM Tris HCl pH8, 100 mM NaCl, 10% glycerol, 1mM PMSF, 1mM DTT, 1 μg/ml bepstatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM sodium butyrate) with or without addition of 150 μM acetyl-CoA. After 1 hour at 30°C, samples were resolved on SDS-PAGE and analyzed by western blot.

***In vitro* deacetylation assay**

500 ng recombinant p65 wild type was *in vitro* acetylated as described previously and then immunoprecipitated. The beads-bound p65 was incubated with 14 pmol of each recombinant SIRT in 100 μl deacetylation buffer (50 mM Tris HCl pH9, 4 mM $MgCl_2$, 0.2 mM DTT, 1 μg/ml bepstatin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) supplemented or not with 1 mM NAD^+ for 30 minutes with constant agitation. Proteins were subsequently resolved in SDS-PAGE and analyzed by western blot. Purified recombinant SIRTs were additionally resolved on SDS-PAGE and stained with coomassie blue.

Acetylation and deacetylation assays in cells

HEK 293T cells were transfected with expression plasmids for p300 and either myc-tagged p65 wild type, the acetylation-deficient mutants or an empty vector, using the calcium phosphate precipitation method. After 23 hours, cells were treated with 10 ng/ml human TNFα for 30 minutes. Then, whole cell extracts were prepared and 40 μg protein was analyzed by SDS-PAGE and western blot. For the deacetylation assay in cells, 0.1 pmol expression plasmid encoding the different sirtuins (wild type or mutants) or empty vector were additionally co-transfected in HEK 293T cells.

Immunostaining

Transfected HEK 293T cells or HeLa cells were stimulated with 10 ng/ml human TNFα for 30 minutes. They were fixed with 4% paraformaldehyde and then permeabilized with 0.2% TritonX-100. Blocking solution (2% BSA and 0.1% TritonX-100 in PBS) was added for 1 hour before the slides were incubated with anti-HA and anti-p65 antibodies (1:250 each in blocking solution), followed by incubation with FITC-labeled anti-mouse and Cy3-labeled anti-rabbit antibodies (1:250 each in blocking solution, Jackson Immunology). The slides were washed, covered with Vectashield mounting solution (Vector laboratories) and visualized using a Leica SP 5 confocal microscope.

ACKNOWLEDGEMENTS

We thank A. Beg, F. Alt, A. Vaquero, K. Chua, I. Horikama and T. Kouzarides for providing useful tools. We would like to thank S. Saccani and G. Natoli for technical suggestions and discussions. We are grateful to the Functional Genomics Center Zurich (FGCZ) for technical support, especially Hubert Rehrauer for his assistance in data generation. We are also grateful to all the members of the Institute of Veterinary Biochemistry and Molecular Biology (University of Zurich, Switzerland) for helpful advice and discussions. This work was supported in part by Swiss National Science Foundation Grant 31-109315 and 31-122421 (to K.R.) and the Kanton of Zurich (to M.O.H.).

REFERENCES

1. **Apostolou, E., and D. Thanos.** 2008. Virus Infection Induces NF-kappaB-dependent interchromosomal associations mediating monoallelic IFN-beta gene expression. Cell **134:**85-96.
2. **Beg, A. A., W. C. Sha, R. T. Bronson, S. Ghosh, and D. Baltimore.** 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature **376:**167-70.
3. **Black, J. C., A. Mosley, T. Kitada, M. Washburn, and M. Carey.** 2008. The SIRT2 deacetylase regulates autoacetylation of p300. Mol Cell **32:**449-55.
4. **Bonizzi, G., M. Bebien, D. C. Otero, K. E. Johnson-Vroom, Y. Cao, D. Vu, A. G. Jegga, B. J. Aronow, G. Ghosh, R. C. Rickert, and M. Karin.** 2004. Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. EMBO J **23:**4202-10.
5. **Buerki, C., K. M. Rothgiesser, T. Valovka, H. R. Owen, H. Rehrauer, M. Fey, W. S. Lane, and M. O. Hottiger.** 2008. Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65. Nucleic Acids Res **36:**1665-80.
6. **Chen, L., W. Fischle, E. Verdin, and W. C. Greene.** 2001. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science **293:**1653-7.
7. **Cheng, H. L., R. Mostoslavsky, S. Saito, J. P. Manis, Y. Gu, P. Patel, R. Bronson, E. Appella, F. W. Alt, and K. F. Chua.** 2003. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci U S A **100:**10794-9.
8. **Denu, J. M.** 2003. Linking chromatin function with metabolic networks: Sir2 family of NAD(+)-dependent deacetylases. Trends Biochem Sci **28:**41-8.

9. **Dryden, S. C., F. A. Nahhas, J. E. Nowak, A. S. Goustin, and M. A. Tainsky.** 2003. Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol Cell Biol **23:**3173-85.
10. **George, A. A., M. Sharma, B. N. Singh, N. C. Sahoo, and K. V. Rao.** 2006. Transcription regulation from a TATA and INR-less promoter: spatial segregation of promoter function. EMBO J **25:**811-21.
11. **Ghosh, S., M. J. May, and E. B. Kopp.** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol **16:**225-60.
12. **Hayden, M. S., and S. Ghosh.** 2008. Shared principles in NF-kappaB signaling. Cell **132:**344-62.
13. **Heltweg, B., T. Gatbonton, A. D. Schuler, J. Posakony, H. Li, S. Goehle, R. Kollipara, R. A. Depinho, Y. Gu, J. A. Simon, and A. Bedalov.** 2006. Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. Cancer Res **66:**4368-77.
14. **Hoffmann, A., and D. Baltimore.** 2006. Circuitry of nuclear factor kappaB signaling. Immunol Rev **210:**171-86.
15. **Hoffmann, A., A. Levchenko, M. L. Scott, and D. Baltimore.** 2002. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science **298:**1241-5.
16. **Huang, B., X. Yang, M. M. Zhou, K. Ozato, and L. F. Chen.** 2008. Brd4 Coactivates Transcriptional Activation of NF-{kappa}B Via Specific Binding to Acetylated RelA. Mol Cell Biol.
17. **Huang, T. T., N. Kudo, M. Yoshida, and S. Miyamoto.** 2000. A nuclear export signal in the N-terminal regulatory domain of IkappaBalpha controls cytoplasmic localization of inactive NF-kappaB/IkappaBalpha complexes. Proc Natl Acad Sci U S A **97:**1014-9.
18. **Huang, Y., P. M. Krein, D. A. Muruve, and B. W. Winston.** 2002. Complement factor B gene regulation: synergistic effects of TNF-alpha and IFN-gamma in macrophages. J Immunol **169:**2627-35.
19. **Kawahara, T. L., E. Michishita, A. S. Adler, M. Damian, E. Berber, M. Lin, R. A. McCord, K. C. Ongaigui, L. D. Boxer, H. Y. Chang, and K. F. Chua.** 2009. SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. Cell **136:**62-74.
20. **Kiernan, R., V. Bres, R. W. Ng, M. P. Coudart, S. El Messaoudi, C. Sardet, D. Y. Jin, S. Emiliani, and M. Benkirane.** 2003. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. J Biol Chem **278:**2758-66.
21. **Langley, E., M. Pearson, M. Faretta, U. M. Bauer, R. A. Frye, S. Minucci, P. G. Pelicci, and T. Kouzarides.** 2002. Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J **21:**2383-96.
22. **Michishita, E., J. Y. Park, J. M. Burneskis, J. C. Barrett, and I. Horikawa.** 2005. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell **16:**4623-35.

23. **Muddasani, P., J. C. Norman, M. Ellman, A. J. van Wijnen, and H. J. Im.** 2007. Basic fibroblast growth factor activates the MAPK and NFkappaB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes. J Biol Chem **282:**31409-21.
24. **North, B. J., B. L. Marshall, M. T. Borra, J. M. Denu, and E. Verdin.** 2003. The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell **11:**437-44.
25. **Oberdoerffer, P., and D. A. Sinclair.** 2007. The role of nuclear architecture in genomic instability and ageing. Nat Rev Mol Cell Biol **8:**692-702.
26. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene **18:**6853-66.
27. **Pandithage, R., R. Lilischkis, K. Harting, A. Wolf, B. Jedamzik, J. Luscher-Firzlaff, J. Vervoorts, E. Lasonder, E. Kremmer, B. Knoll, and B. Luscher.** 2008. The regulation of SIRT2 function by cyclin-dependent kinases affects cell motility. J Cell Biol **180:**915-29.
28. **Perez, P., S. A. Lira, and R. Bravo.** 1995. Overexpression of RelA in transgenic mouse thymocytes: specific increase in levels of the inhibitor protein I kappa B alpha. Mol Cell Biol **15:**3523-30.
29. **Perkins, N. D.** 2006. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene **25:**6717-30.
30. **Perkins, N. D., L. K. Felzien, J. C. Betts, K. Leung, D. H. Beach, and G. J. Nabel.** 1997. Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. Science **275:**523-7.
31. **Perrod, S., M. M. Cockell, T. Laroche, H. Renauld, A. L. Ducrest, C. Bonnard, and S. M. Gasser.** 2001. A cytosolic NAD-dependent deacetylase, Hst2p, can modulate nucleolar and telomeric silencing in yeast. EMBO J **20:**197-209.
32. **Pfaffl, M. W., G. W. Horgan, and L. Dempfle.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res **30:**e36.
33. **Sauve, A. A., C. Wolberger, V. L. Schramm, and J. D. Boeke.** 2006. The biochemistry of sirtuins. Annu Rev Biochem **75:**435-65.
34. **Sterner, D. E., and S. L. Berger.** 2000. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev **64:**435-59.
35. **Taylor, D. M., M. M. Maxwell, R. Luthi-Carter, and A. G. Kazantsev.** 2008. Biological and potential therapeutic roles of sirtuin deacetylases. Cell Mol Life Sci **65:**4000-18.
36. **Teferedegne, B., M. R. Green, Z. Guo, and J. M. Boss.** 2006. Mechanism of action of a distal NF-kappaB-dependent enhancer. Mol Cell Biol **26:**5759-70.
37. **Vaquero, A., M. B. Scher, D. H. Lee, A. Sutton, H. L. Cheng, F. W. Alt, L. Serrano, R. Sternglanz, and D. Reinberg.** 2006. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. Genes Dev **20:**1256-61.
38. **Vaquero, A., R. Sternglanz, and D. Reinberg.** 2007. NAD+-dependent deacetylation of H4 lysine 16 by class III HDACs. Oncogene **26:**5505-20.

39. **Westphal, C. H., M. A. Dipp, and L. Guarente.** 2007. A therapeutic role for sirtuins in diseases of aging? Trends Biochem Sci **32:**555-60.
40. **Yamamoto, H., K. Schoonjans, and J. Auwerx.** 2007. Sirtuin functions in health and disease. Mol Endocrinol **21:**1745-55.
41. **Yang, X. D., B. Huang, M. Li, A. Lamb, K. N.L., and L. F. Chen.** 2009. Negative regulation of NF-kappaB action by Set9-mediated lysine methylation of the RelA subunit EMOB J**:**Advanced online publicaiton.
42. **Yang, X. J., and E. Seto.** 2007. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene **26:**5310-8.
43. **Yeung, F., J. E. Hoberg, C. S. Ramsey, M. D. Keller, D. R. Jones, R. A. Frye, and M. W. Mayo.** 2004. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J **23:**2369-80.
44. **Zhong, H., M. J. May, E. Jimi, and S. Ghosh.** 2002. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell **9:**625-36.

FIGURE LEGENDS

Figure 1. Site-specific acetylation of p65 regulates the expression of distinct genes. Gene induction of *Cfb* **(A)***, Mpa2l* **(B)***, Mmp10* **(C)** and *Mmp13* **(D)** in a TNFα-dependent manner, as measured by real-time RT-PCR, from the following complemented cell lines: wild type (green), K310R (blue), K314/315R (brown), KTR (red) and pTV (black). Samples were normalized to *Rps6* and *CanX* expression levels, and expressed as fold increase relative to wild type unstimulated. Two biological replicates were included. Shown are the means ± SD of three independent runs.

Figure 2. Acetylated p65 is recruited to the promoter region of regulated genes upon TNFα stimulation. (A-B) Characterization of specific antibodies against p65 acetylated at lysine 310, 314 or 315. **(A)** Purified recombinant p65 wild type and the acetylation-deficient mutants were incubated with recombinant p300 in the presence (+) or absence (-) of acetyl CoA. Proteins were resolved on SDS-PAGE and analyzed by western blot using the indicated antibodies. **(B)** HEK 293T cells were transfected with p65 wild type or mutants, with (+) or without (-) p300 co-transfection. Acetylation of p65 at specific lysines was assessed by western blot using the specific antibodies. * indicates a non-specific band. Chromatin immunoprecipitation analysis of p65 **(C)** or acetylated p65 at

lysine 310 or 314 **(D)** from *p65*(+/+) MEFs kept unstimulated or treated with TNFα for 20 or 180 minutes. Chromatin from *p65*(-/-) MEFs stimulated with TNFα for 180 minutes was used as negative control. Recruitment to the indicated promoters was analyzed. Occupancy to *IP-10* and *Glucagon* promoters was assessed as positive and negative control, respectively. Samples were normalized to input chromatin and expressed as % input. The result is representative of three independent experiments. Mean values ± SD of three independent runs are shown.

Figure 3. SIRT2 deacetylates p65 at lysines 310, 314 and 315. (A). Endogenous p65 is rapidly and possibly transiently acetylated in the nucleus upon TNFα stimulation *in vivo*. Wild type complemented MEFs were treated with TNFα for the indicated time periods. p65 was immunoprecipitated from nuclear extracts and analyzed by western blot using anti-acetyl K310 antibody. PARP1 from 5% input was used as nuclear loading control. **(B)** Overexpressed SIRT1 and SIRT2 deacetylate p65 in HEK 293T cells. HEK 293T cells were transfected with the expression plasmids for the indicated his-tagged SIRTs along with p300 and myc-tagged p65. Whole cell extracts were prepared after 30 minutes of TNFα stimulation and analysed by western blot. **(C)** Deacetylase activity of SIRT1 and SIRT2 is needed for p65 deacetylation in cells. SIRT1 and SIRT2 wild type and the enzymatic inactive mutants were overexpressed in HEK 293T cells together with p300 and myc-tagged p65. The acetylation status of p65 after 30 minutes of TNFα stimulation was assessed with the indicated antibodies. **(D)** *In vitro* acetylated p65 wild type was incubated with recombinant sirtuins in the presence (+) or absence (-) of NAD^+. Western blot analysis shows *in vitro* deacetylation of lysines 310, 314 and 315 by SIRT1 and SIRT2. **(E)** Endogenous p65 is hyperacetylated in *Sirt2*(-/-) cells. MEFs from the indicated genotypes were pre-treated with the HDAC inhibitors (HDACi) TSA and Nam for 30 minutes and then stimulated with TNFα for 1 hour before preparing whole cell extracts. Extracts were analyzed by western blot using anti-acetyl K310 antibody. The membrane was reprobed for p65, and tubulin was used as loading control. * indicates a non-specific band.

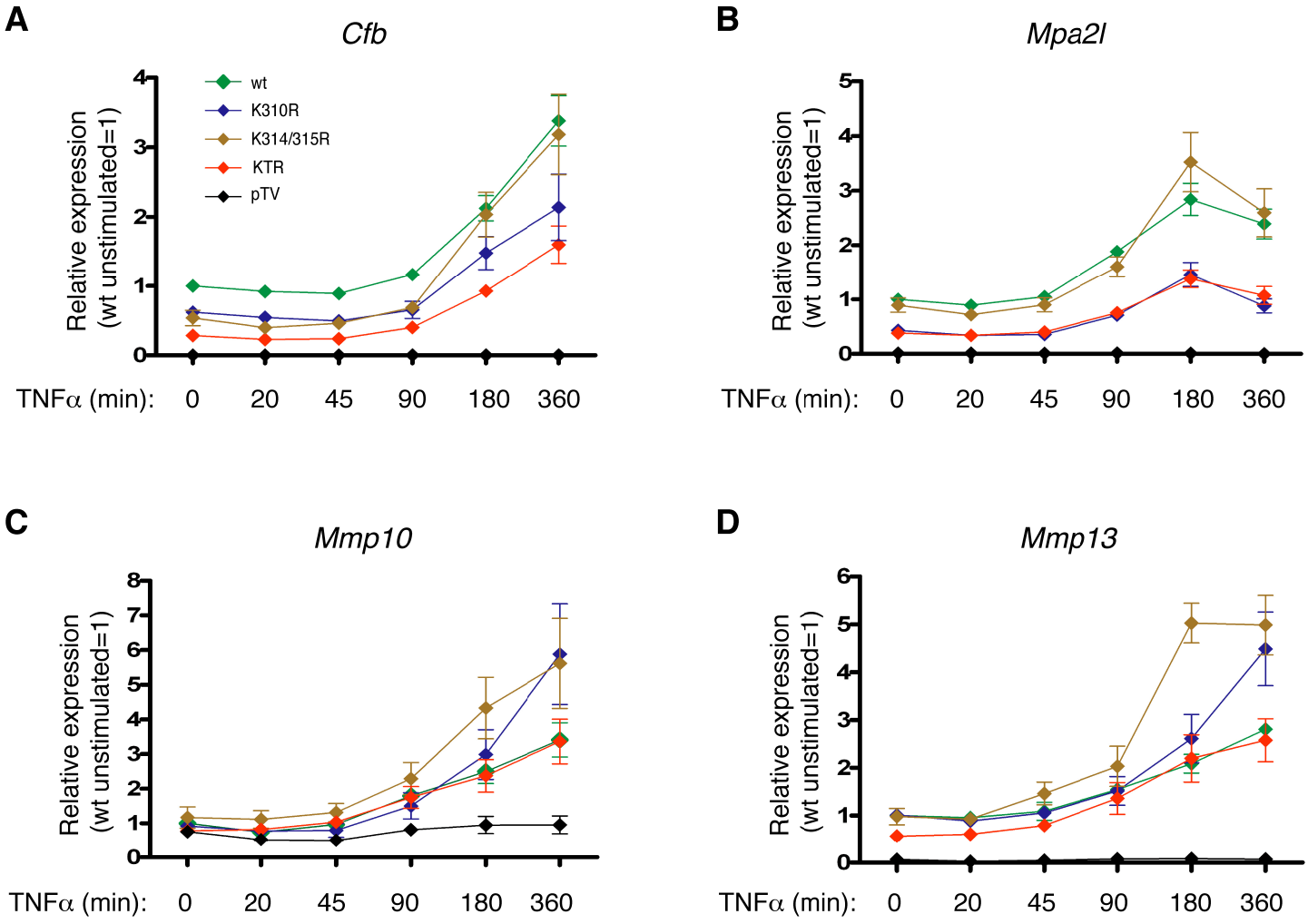
Figure 4. SIRT2 deacetylates p65 in the cytoplasm. (A) p65 and SIRT2 interact in the cytoplasm. Cytoplasmic (C) and nuclear (N) extracts were generated from unstimulated Jurkat T-cells, endogenous SIRT2 was immunoprecipitated and co-immunoprecipitated p65 was detected by western blot (upper panels). Levels of SIRT2, p65, PARP1 as nuclear control and tubulin as loading control from 5% input are shown in the lower panels. **(B)** p65 translocates to the nucleus in response to TNFα stimulation, while SIRT2 stays in the cytoplasm. HeLa cells were transfected with HA-tagged SIRT2 and stimulated with TNFα for the indicated time points, followed by immunofluorescence detection of p65 and HA-SIRT2. Dapi staining shows the nucleus. Subcellular localization of p65 and HA-SIRT2 was analysed with a confocal microscope. **(C)** HEK 293T cells were transfected with HA-tagged SIRT2 wild type, NLS mutant or NLSΔNES mutant. After 30 minutes of TNFα stimulation, cells were fixed and immunostained with anti-HA antibody to display cellular localization of SIRT2 wild type and mutant proteins, assessed with a confocal microscope. **(D)** p65 has a decreased deacetylation when nuclear SIRT2 mutant is expressed. The same constructs as in (C) were overexpressed in HEK 293T cells along with p65 wild type and p300, followed by 30 minutes of TNFα stimulation, whole cell extracts preparation and western blot analysis with the indicated antibodies.

Figure 5. Lack of SIRT2 increases TNFα-induced NF-κB-dependent transcriptional activation. Gene induction of *Mpa2l* **(A)**, *Mmp13* (**B**) and *RELA* (**C**) in *Sirt2*(+/+) and *Sirt2*(-/-) MEFs after 3 hours of TNFα stimulation, as measured by real-time RT-PCR. At least two biological replicates were analyzed. Shown are the means ± SD of three independent runs.

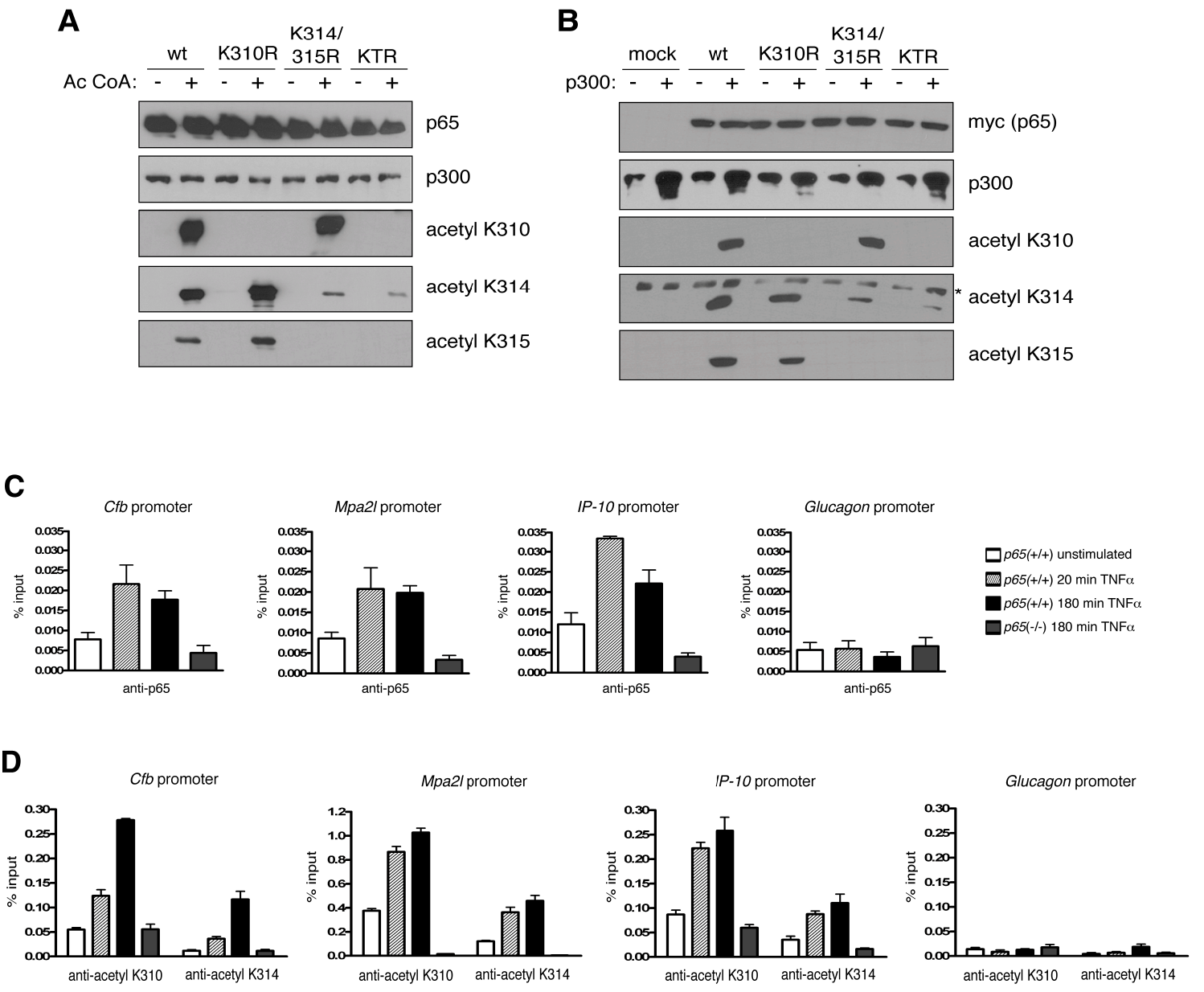
Figure 6. p65 is acetylated in the nucleus to modulate gene expression and deacetylated in the cytoplasm by SIRT2. In unstimulated cells, p65 is in a complex with SIRT2 in the cytoplasm. Upon TNFα stimulation, the complex dissociates and p65/p50 heterodimers translocate to the nucleus, where they bind to promoters of regulated genes. Co-activator p300 binds to p65 and acetylates it at lysines 310, 314 and

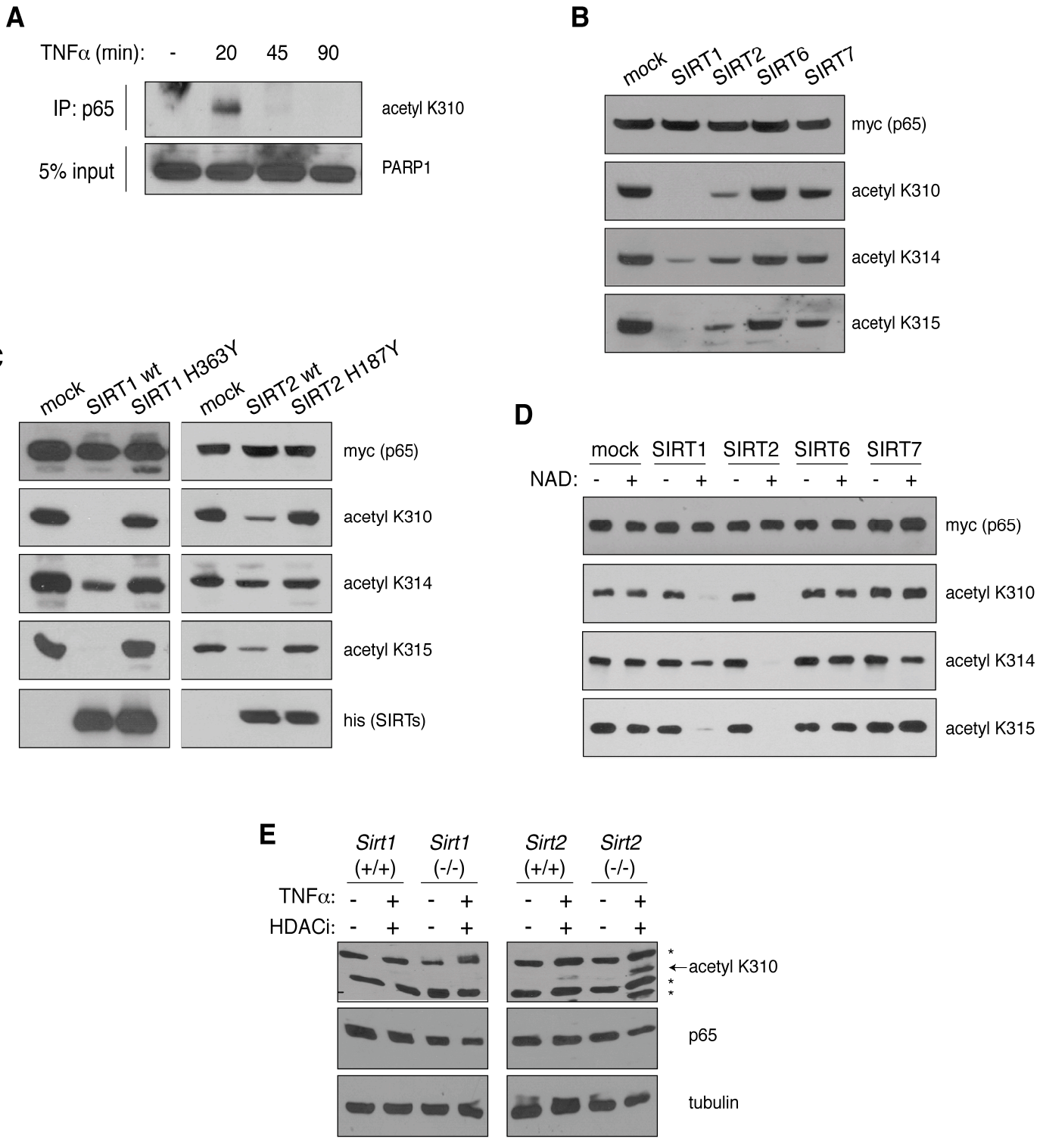
315 to fine tune gene expression. Upon termination of the NF-κB response, p65/p50 heterodimers shuttle back to the cytoplasm, where p65 is deacetylated by SIRT2.

Rothgiesser et al., Figure 1

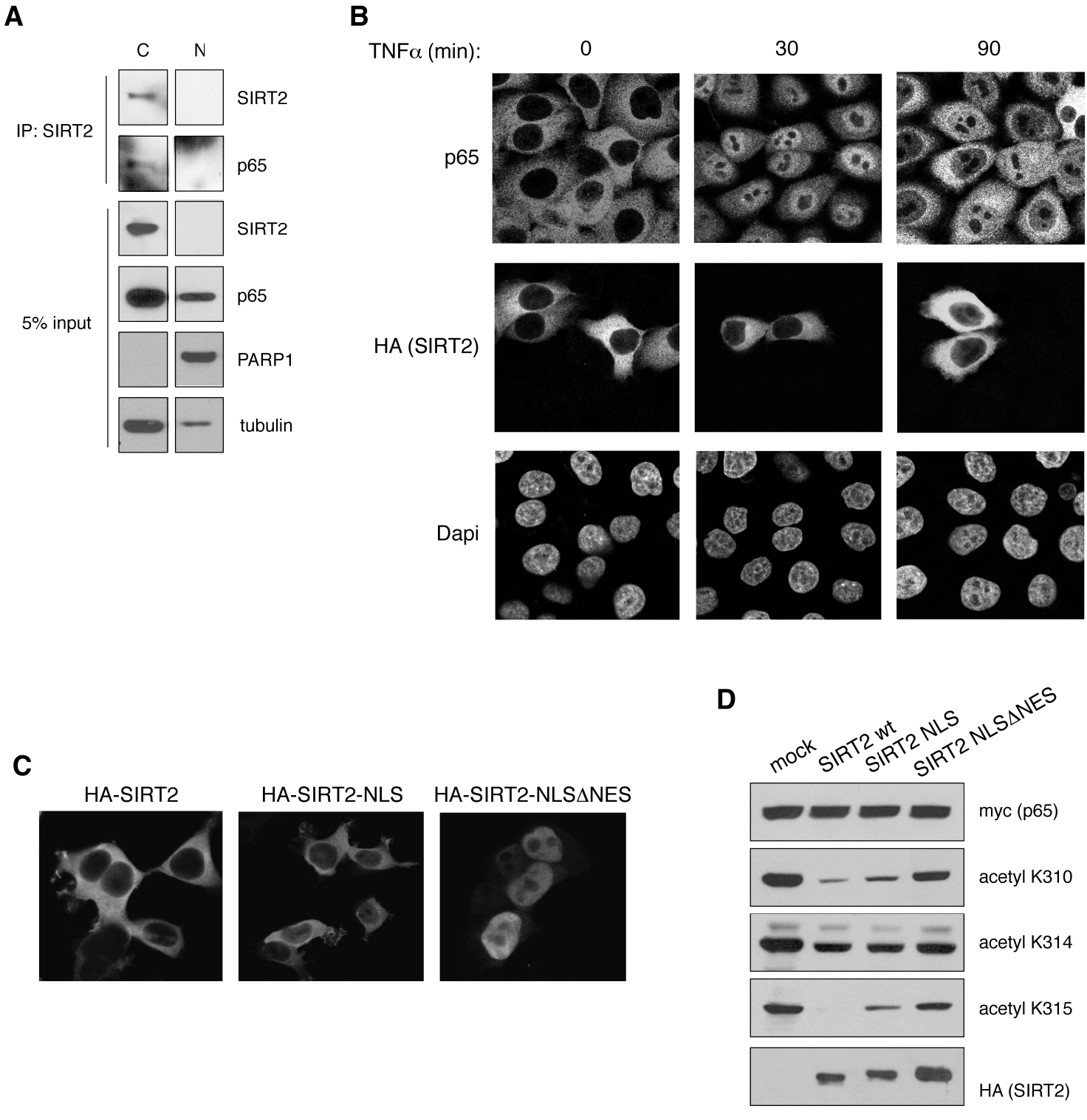


Rothgiesser et al., Figure 2

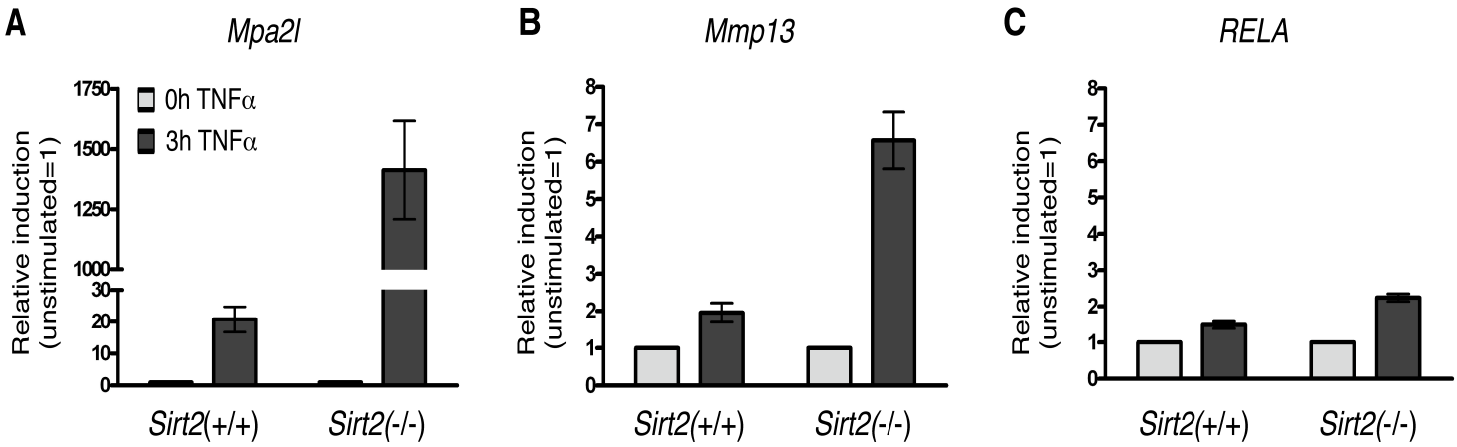



A
TNFα (min): - 20 45 90
IP: p65
acetyl K310
5% input
PARP1
B
mock
SIRT1
SIRT2
SIRT6
SIRT7
myc (p65)
acetyl K310
acetyl K314
acetyl K315
C
mock
SIRT1 wt
SIRT1 H363Y
mock
SIRT2 wt
SIRT2 H187Y
myc (p65)
acetyl K310
acetyl K314
acetyl K315
his (SIRTs)
D
mock
SIRT1
SIRT2
SIRT6
SIRT7
NAD: - + - + - + - + - +
myc (p65)
acetyl K310
acetyl K314
acetyl K315
E
Sirt1 (+/+)
Sirt1 (-/-)
Sirt2 (+/+)
Sirt2 (-/-)
TNFα: - + - + - + - +
HDACi: - + - + - + - +
acetyl K310
p65
tubulin

Rothgiesser et al., Figure 4



Rothgiesser et al., Figure 5



Rothgiesser et al., Figure 6

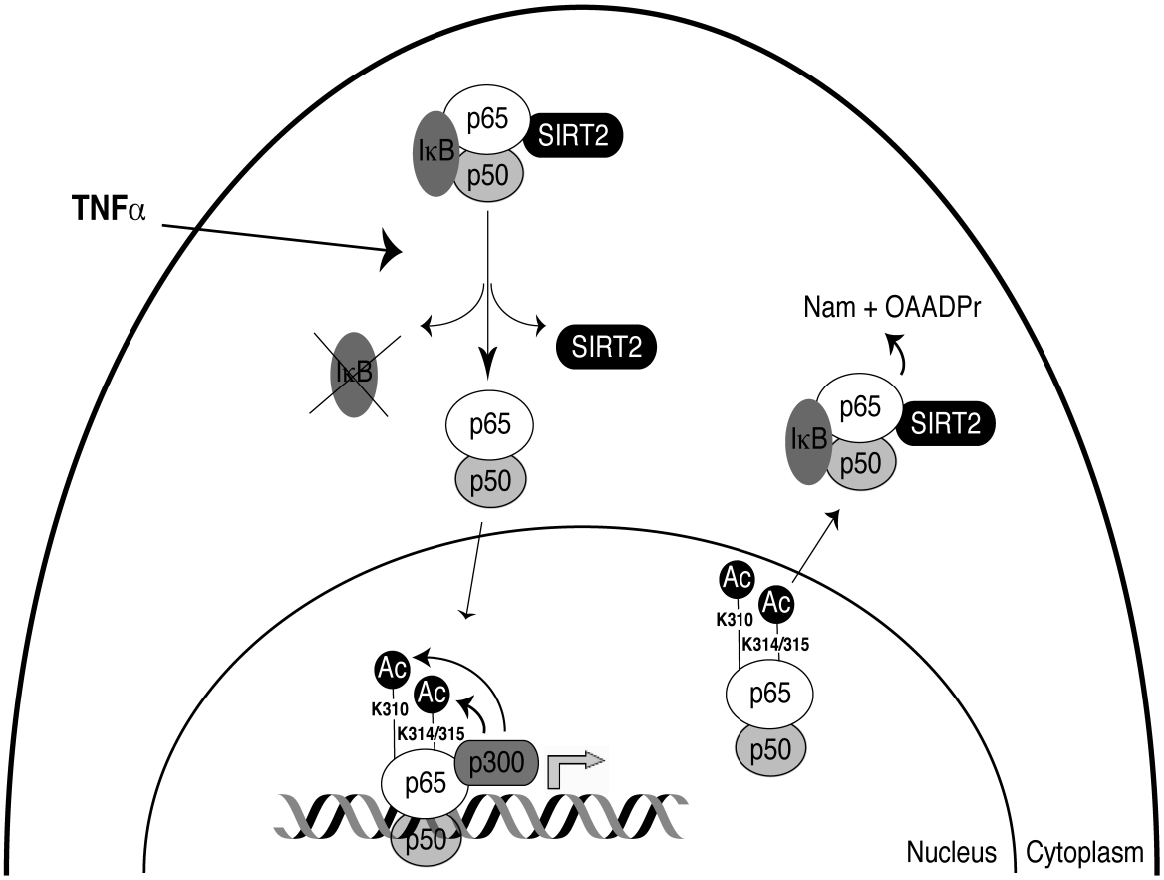


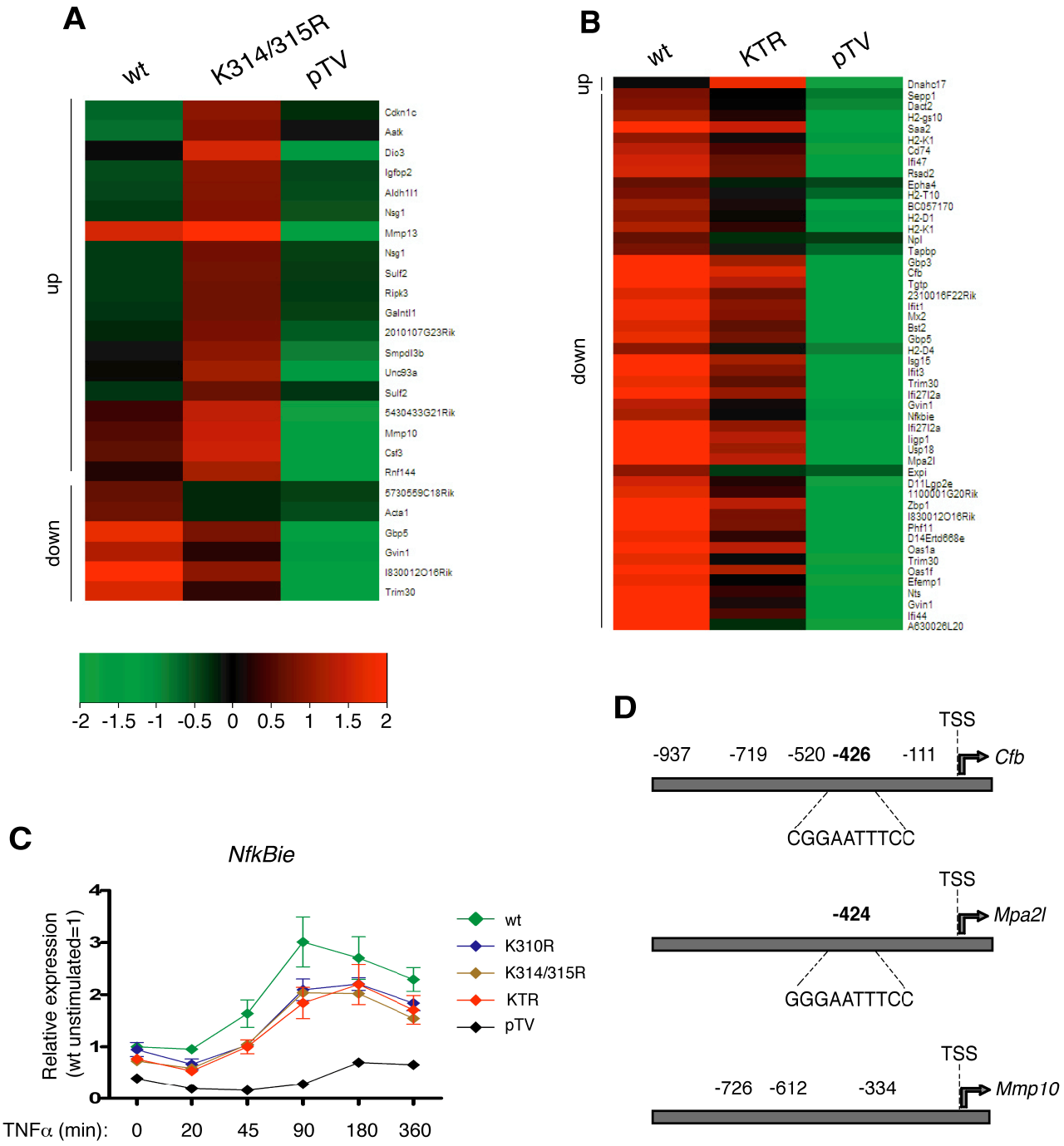
Figure S1. (A-B) Heat maps showing the gene expression profiles of K314/315R cells **(A)** or KTR cells **(B)** compared to wild type control at 3 hours after TNFα stimulation using whole mouse genome arrays. Each row represents a single gene, and each column represents a different cell line. Red or green represents up- or downregulation of genes relative to the mean on each row, respectively. Mean data from at least two biological replicates is displayed. Only genes upregulated in wild type cells compared to pTV control were taken into account (>2-fold, p-value <0.05). From these genes, only the ones with significant changes in expression levels (> 1.8-fold or < 0.556-fold, p-value < 0.05) between wild type and mutant cells are shown. **(C)** mRNA levels of *NfκBie* relative to wild type unstimulated sample, as measured by qPCR. Cells were stimulated with TNFα for the indicated time periods. Data was normalized to *Rps6* and *CanX* expression levels. Shown are the means ± SD of three independent runs. **(D)** Promoters of *Cfb, Mpa2l* and *Mmp10* have putative κB sites. Schematic representation of putative κB sites found in the indicated promoter regions. The distance in bp relative to the transcription start site (TSS) of every putative κB site is shown, as well as the sequence from the κB site chosen for analysis by ChIP.

Figure S2. (A) Different myc-tagged HDACs were overexpressed in HEK 293T cells together with p300 and myc-tagged p65 and analyzed by western blot using anti-myc antibody (left panel) or anti-acetyl K310 antibody (right panel). **(B)** Western blot against his tag of overexpressed SIRT proteins to show expression levels of these proteins in HEK 293T cells. **(C)** Recombinant SIRT proteins used in the *in vitro* deacetylation assay were resolved by SDS-PAGE and stained with coomassie blue. **(D)** Western blot analysis of untreated samples showing no compensatory effect between SIRT1 and SIRT2 in the knock out cells. Tubulin served as loading control.

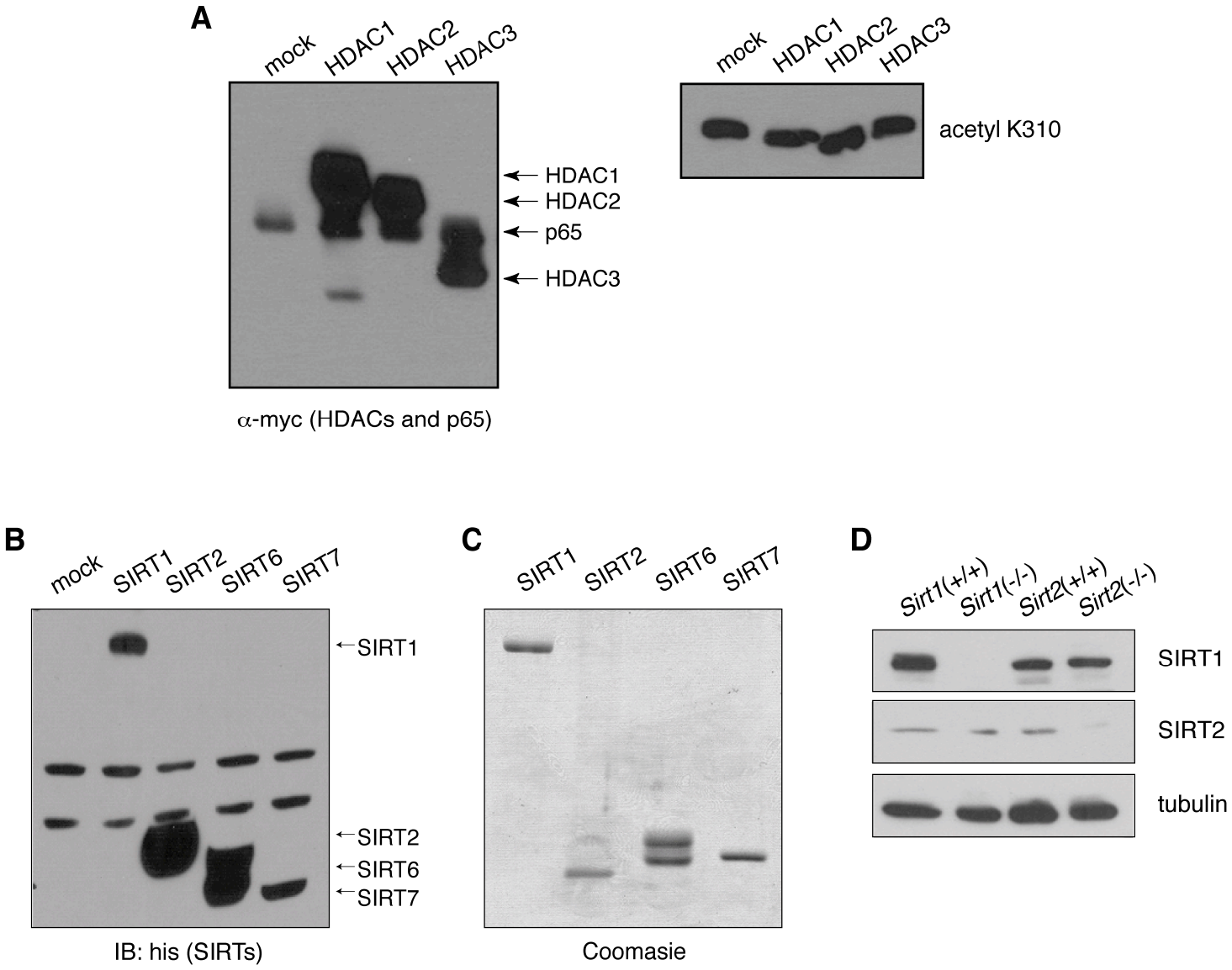
Table SI. Lists of up- and downregulated genes in K314/315R cell line compared to wild type control after 3 hours of TNFα treatment, as measured by whole genome arrays. Average values from at least two biological replicates are shown.

Table SII. Lists of differentially regulated genes in KTR versus wild type control at 3 hours TNFα stimulation analyzed by microarrays. Average values from at least two biological replicates are shown.

Rothgiesser et al., Figure S1



Rothgiesser et al., Figure S2



Rothgiesser et al., Table SI

RefSeq RNA	Gene symbol	Gene name	Fold change	P-value
NM_007377	Aatk	Apoptosis-associated tyrosine kinase	2.995	7.21E-29
NM_009876	Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	2.979	1.99E-09
NM_172119	Dio3	Deiodinase, iodothyronine type III	2.685	2.31E-07
NM_027406	Aldh1l1	Aldehyde dehydrogenase 1 family, member L1	2.39	1.35E-09
NM_008342	Igfbp2	Insulin-like growth factor binding protein 2	2.381	0.000196
NM_010942	Nsg1	Neuron specific gene family member 1	2.254	0.000001
NM_008607	Mmp13	Matrix metallopeptidase 13	2.222	4.57E-07
NM_010942	Nsg1	Neuron specific gene family member 1	2.119	0.000013
NM_028072	Sulf2	Sulfatase 2	2.1	0.000004
NM_027251	2010107G23Rik	RIKEN cDNA 2010107G23 gene	2.057	1.81E-07
NM_019955	Ripk3	Receptor-interacting serine-threonine kinase 3	2.047	5.51E-10
NM_133888	Smpdl3b	Sphingomyelin phosphodiesterase, acid-like 3B	2.04	8.17E-19
NM_001081421	Galntl1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 1	2.005	0.000414
NM_028072	Sulf2	Sulfatase 2	1.956	1.26E-07
NM_199252	Unc93a	Unc-93 homolog A (C. elegans)	1.938	0.000978
XM_283765	5430433G21Rik	RIKEN cDNA 5430433G21 gene	1.915	0.00025
NM_080563	Rnf144	Ring finger protein 144	1.83	0.006
NM_019471	Mmp10	Matrix metallopeptidase 10	1.829	0.000263
NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	1.807	0.000516

RefSeq RNA	Gene symbol	Gene name	Fold change	P-value
NM_029000	Gvin1	GTPase, very large interferon inducible 1	0.501	0.005
NM_009099	Trim30	Tripartite motif protein 30	0.506	0.036
AK077243	I830012O16Rik	RIKEN cDNA I830012O16 gene	0.509	0.008
NM_009606	Acta1	Actin, alpha 1, skeletal muscle	0.511	4.48E-14
NM_153564	Gbp5	Guanylate binding protein 5	0.521	0.000007
NM_028872	5730559C18Rik	RIKEN cDNA 5730559C18 gene	0.523	8.43E-07

Rothgiesser et al., Table SII

RefSeq RNA	Gene symbol	Gene name	Fold change	P-value
XM_126677	Dnahc17	Dynein, axonemal, heavy chain 17	3.081	2.80E-45

RefSeq RNA	Gene symbol	Gene name	Fold change	P-value
XR_001627	A630026L20	Hypothetical protein A630026L20	0.188	9.40E-30
NM_029000	Gvin1	GTPase, very large interferon inducible 1	0.26	8.04E-09
NM_024435	Nts	Neurotensin	0.262	5.50E-10
NM_133871	Ifi44	Interferon-induced protein 44	0.282	0.001
NM_146015	Efemp1	Epidermal growth factor-containing fibulin-like extracellular matrix protein 1	0.286	2.31E-20
NM_145153	Oas1f	2'-5' oligoadenylate synthetase 1F	0.349	0.000045
NM_199015	D14Ertd668e	DNA segment, Chr 14, ERATO Doi 668, expressed	0.368	0.000005
NM_145211	Oas1a	2'-5' oligoadenylate synthetasre 1A	0.369	0.001
NM_172603	Phf11	PHD finger protein 11	0.371	3.08E-07
NM_009099	Trim30	Tripartite motif protein 30	0.378	0.001
NM_183249	1100001G20Rik	RIKEN cDNA 1100001G20 gene	0.395	8.10E-07
NM_030150	Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58	0.413	0.000006
NM_021394	Zbp1	Z-DNA binding protein 1	0.419	0.000443
NM_008690	Nfkbie	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	0.422	0.000005
XM_001000862	I830012O16Rik	RIKEN cDNA I830012O16 gene	0.422	0.002
NM_021792	Iigp1	Interferon inducible GTPase 1	0.427	0.000001
NM_194336	Mpa2l	Macrophage activation 2 like	0.435	0.039
NM_007969	Expi	Extracellular proteinase inhibitor	0.436	0.000025
NM_029000	Gvin1	GTPase, very large interferon inducible 1	0.438	0.000024
NM_009099	Trim30	Tripartite motif protein 30	0.454	0.000002
NM_011909	Usp18	Ubiquitin specific peptidase 18	0.458	0.002
NM_029803	Ifi27l2a	Interferon, alpha-inducible protein 27 like 2A	0.46	0.000163
NM_029803	Ifi27l2a	Interferon, alpha-inducible protein 27 like 2A	0.462	0.000471
NM_008200	H2-D4	Histocompatibility 2, D region locus 4	0.483	0.000006
NM_010501	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	0.488	0.000741
NM_153564	Gbp5	Guanylate binding protein 5	0.493	0.000843
NM_013606	Mx2	Myxovirus (influenza virus) resistance 2	0.509	0.000002
NM_198095	Bst2	Bone marrow stromal cell antigen 2	0.511	0.000046
NM_009318	Tapbp	TAP binding protein	0.511	0.004
NM_172777	BC057170	cDNA sequence BC057170	0.519	0.000028
NM_018734	Gbp3	Guanylate binding protein 3	0.52	0.000867
NM_001001892	H2-K1	Histocompatibility 2, K1, K region	0.522	7.26E-07
NM_011579	Tgtp	T-cell specific GTPase	0.522	0.000163
NM_008198	Cfb	Complement factor B	0.523	0.000011
NM_010395	H2-T10	Histocompatibility 2, D region locus 1	0.527	0.000001
NM_007936	Epha4	Eph receptor A4	0.528	0.001
NM_010545	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	0.532	2.94E-09
NM_008331	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	0.532	0.002
NM_001001892	H2-K1	Histocompatibility 2, K1, K region	0.533	5.43E-08
NM_028749	Npl	N-acetylneuraminate pyruvate lyase	0.533	0.000004
NM_010380	H2-D1	Histocompatibility 2, D region locus 1	0.533	0.000007
NM_015783	Isg15	ISG15 ubiquitin-like modifier	0.534	0.008
NM_173743	2310016F22Rik	RIKEN cDNA 2310016F22 gene	0.535	0.000166
NM_011314	Saa2	Serum amyloid A 2	0.536	3.53E-07
NM_008330	Ifi47	Interferon gamma inducible protein 47	0.542	0.000013
NM_172826	Dact2	Dapper homolog 2, antagonist of beta-catenin (xenopus)	0.544	0.008
NM_021384	Rsad2	Radical S-adenosyl methionine domain containing 2	0.547	0.005
NM_001143689	H2-gs10	MHC class I like protein GS10	0.55	0.000007
NM_009155	Sepp1	Selenoprotein P, plasma, 1	0.552	1.58E-14

CARM1 BUT NOT ITS ENZYMATIC ACTIVITY IS REQUIRED FOR TRANSCRIPTIONAL COACTIVATION OF NF-κB-DEPENDENT GENE EXPRESSION

Sandrine Jayne, Karin M. Rothgiesser and Michael O. Hottiger
Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich
Winterthurerstrasse 190, 8057 Zurich, Switzerland.
Running head: CARM1 activity is dispensable for NF-κB
Address correspondence to: Phone: +41-44-635 54 74. Fax: +41-44-635 68 40.
E-mail: hottiger@vetbio.uzh.ch

CARM1 belongs to the protein arginine methyltransferase family. It was reported to methylate histone as well as non-histone proteins and thus to be involved in transcriptional activation and mRNA degradation/stability. Here we report the genetic complementation of *carm1*-/- cells with wild type CARM1 or an enzymatic inactive mutant of CARM1 to investigate the requirement of CARM1 and its enzymatic activity for NF-κB-dependent gene expression. Using custom micro array and real time RT-PCR, we could define a subset of NF-κB target genes that required CARM1 for their proper expression. Although several TNFα and PMA/Ionomycin induced NF-κB target genes are CARM1-dependent, CARM1 enzymatic activity was dispensable for gene expression. Interestingly, CARM1 was not required for the stimuli-dependent recruitment of NF-κB, suggesting that CARM1 is rather contributing in protein complex formation. Together, our results confirm the importance of CARM1 as transcriptional cofactor without the involvement of its catalytic activity.

The widely expressed and inducible transcription factor, nuclear factor-kappaB (NF-κB), regulates the expression of many effectors and inducers that impact immune and inflammatory responses, apoptosis, differentiation, cell proliferation and transformation (1,2). NF-κB/REL belongs to a family of five proteins, which share a conserved 300 amino acids region within their amino-termini, known as the Rel-homology domain (RHD) that mediates dimerization, nuclear translocation, DNA-binding and interaction with inhibitory proteins (IκBs) (1). The best-studied NF-κB heterodimer is composed of RelA/p65 and p50/p105(NF-κB1) members. In most differentiated unstimulated cells, inactive NF-κB is sequestered in the cytoplasm by its physical association with inhibitory proteins IκBs. Treatment of cells with extra-cellular stimuli such as cytokines, bacterial lipopolysaccharides (LPS) or potent oxidants leads to the rapid degradation of the IκBs by the 26S proteasome pathway (3). Consequently, NF-κB accumulates in the nucleus, binds to specific κB DNA consensus sequences and activates specific target genes.

Gene expression requires the combinatorial and synchronized action of many transcriptional cofactors, which act as recruiting platform either directly by protein-protein interactions with secondary coactivators or via their enzymatic activity by modifying histone tails or coactivators (4,5). Specialized modules of coactivator proteins recognize modified residues on histone tails or coactivators (6,7). Like other transcription factors, NF-κB-dependent gene expression required many transcriptional coactivators, among which the histone acetyltransferases p300/CBP (CREB-Binding Protein), pCAF (p300/CBP-Associated Factor), the p160 family of coactivator proteins, PARP1 (poly(ADP)-ribose polymerase1), PRMT1 (Protein Arginine Methyltransferase 1) and CARM1/PRMT4 (Coactivator-Associated Arginine Methyltransferase 1/ Protein Arginine Methyltransferase 4) (8-11).

CARM1 belongs to the protein arginine methyltransferase family, which contains 10 members, all sharing a specific signature motif (12). CARM1 acts as a coactivator of many transcription factors (11,13-15). Moreover, it was reported to methylate histone H3 at arginine 17 (H3R17), which correlates with transcriptional activation (16), and a unique set of non-histone proteins (17-24). Furthermore, besides its role in transcription regulation, CARM1 affects also mRNA stability/degradation of some specific mRNAs bearing AU-rich elements (ARE) at their 3'-untranslated regions (UTR) by methylating the protein HuD (23). CARM1 has also been implicated in the epigenetic programming of early

embryos (25). Cells from *carm1-/-* embryos have defective estrogen receptor, c-Fos, PPARγ and NF-κB pathways (11,15,26,27). The CARM1 crystal structure was solved by two groups, providing insight into its mechanism of action (28,29).

Despite intensive investigations regarding the function of CARM1 as transcriptional co-activator, the contribution of the protein itself or its enzymatic activity is still unclear and even controversial. In this report, we genetically complemented *carm1-/-* cells with CARM1 wild type or an enzymatic inactive CARM1 mutant to subsequently analyze expression of NF-κB target genes. Our experiments strongly provide evidence that CARM1 itself but not its enzymatic activity is required for NF-κB-dependent gene expression.

Experimental Procedures

Cell culture

Carm1-/- mouse embryonic fibroblasts (kindly provided by Dr. Bedford (University of Texas)) (26) and the complemented *Carm1-/-(+wt)* and *Carm1-/-(+mut)* cells were maintained in DMEM (Invitrogen) containing 10% FCS, 100 units/ml penicillin/streptomycin and non-essential amino acids (GIBCO). Before stimulation with TNFα (10ng/ml) (Sigma) or phorbol-12-myristate-13-acetate (PMA) (0.11 mg/ml)(Sigma)/Ionomycin (0.2μM) (Sigma), cells were grown for 12 hours in 2% FCS-containing medium. Actinomycin D (Sigma) was used at a final concentration of 5μg/ml.

Lentiviral complementation of carm1-/- cells

Virus production and transduction of *carm1-/-* cells was performed as described in (30). Briefly, HEK293T cells were transfected with 3.5 μg of the envelope plasmid, 6.5 μg of packaging plasmid, and 10 μg of pRRL-FlagCARM1 wild type, FlagCARM1 E267Q mutant. After 24 h the viral supernatant was harvested and used to infect *carm1-/-* MEFs. Thirty-six hours post infection cells were split into selective medium containing 2.5 mg/ml Blasticidin (Sigma). Expression of recombinant proteins in the complemented cells was screened by western blot analysis. Pools of cells were used for further analysis.

Proteins

N-terminal 6xhis tagged CARM1 was expressed in Sf21 insect cells using baculovirus technology and purified in batch through Ni-NTA ProBond Resin (Invitrogen). GST-PABP was expressed in *E.coli* Bl21DE3 strain. The soluble protein was purified in batch through Glutathione Sepharose beads (Amersham Biosciences).

Antibodies

Anti-CARM1 (07-080) was purchased from Millipore, anti-PRMT1 (ab3768) from Abcam, anti-RelA/p65 (C20, sc372) and anti-tubulin (TU-02, sc-8035) from Santa-Cruz Biotechnologies and anti-Flag M2 from Sigma.

Plasmids

The pRRL lentivirus transfer vector used in this study has been previously described (30). The enzymatic mutant form (E267Q) of CARM1 was created by site directed mutagenesis as described in (31) and verified by sequencing.

In vitro methylation assay

Assay using whole cell extracts was performed as described in (26).

RNA isolation, Reverse-transcription

Total RNAs were isolated from cells using Total RNA Isolation kit (Agilent Technologies) following manufacturer's instructions. Purified RNAs were reverse-transcribed using Retroscript kit (Ambion).

Real-time PCR

Rotor-Gene3000A (Corbett) was used to perform the real-time PCR reactions. Real-time PCR was performed using SensimixPlusSYBR (Quantace) and specific primers encompassing the regions used in the micro array analysis (sequences available in suppl. table). The REST program was applied for gene expression analysis (32).

Micro array

RNA quality was assessed with the RNA 6000 Nano kit using the Bioanalyzer 2001 (Agilent Technologies). Purified RNAs were converted into double-stranded cDNA and transcribed into AlexaFluor647/AlexaFluor555 labeled cRNA using the AminoAllyl MessageAmp™ II aRNA Amplification kit (Ambion). Dye incorporation was measured on the ND-1000 Spectrophotometer (NanoDrop Technologies).

Activated CodeLink slides (GE Healthcare) were printed at the Functional Genomics Center Zurich (Switzerland). They contain 350 probes for previously identified or putative NF-κB-dependent genes and 174 probes for control genes. The probes were 60 nucleotides long. Hybridizations were performed at the Functional Genomics

Center Zürich (Switzerland). Equal amount of dye-labeled aRNAs was hybridized for 16 hours. Slides were scanned using the Agilent DNA micro array scanner and the scans were quantified with the Agilent Feature Extraction software. Data analysis was performed with GeneSpring software (Silicon Genetics).

Chromatin immunoprecipitation

ChIP on cross-linked chromatin was performed as described in (11). All ChIP experiments were performed several times on independent chromatin preparations. Immunoprecipitated DNA and input DNA were analyzed by real-time PCR using specific primers (sequences available in suppl. table). Microarray data have been deposited in the ArrayExpress under accession numbers A-MEXP-1502 (array design) and E-MEXP-2020 (experiment data).

RESULTS

Genetic complementation of carm1-/- cells with wild type and enzymatic inactive CARM1.

In a previous study, we could show that CARM1 is a transcriptional coactivator of NF-κB-dependent gene expression (11). To assess the contribution of the presence of CARM1 and of its methyltransferase activity for the transcriptional coactivation function, we genetically complemented *carm1-/-* MEF cells by retroviral transduction, either with wild type CARM1 (*carm1-/-(+wt)*) or with the enzymatic inactive E267Q mutant of CARM1 (*carm1-/-(+mut)*) (31).

Protein expression levels of the complemented CARM1 (wt and mut) were comparable to the CARM1 expression levels in a rescued cell line, in which CARM1 expression was restored by transfection of flp-recombinase (26) (Fig.1A). Complementing cells did not alter the expression level of PRMT1 protein or of p65 (Fig.1A and data not shown). To confirm that CARM1 in *carm1-/-(+mut)* cells was indeed enzymatic inactive, we performed a radioactively labeled *in vitro* methylation assay using whole cell extracts from the different complemented cell lines and GST-PABP as a CARM1 specific substrate. Arginine methylation was controlled with a recombinant purified active CARM1 as positive control. While GST-PABP was *in vitro* arginine-methylated in the presence of the recombinant protein and in the presence of cell extracts from *carm1-/-(+wt)* cells, no methylation of the target protein was observed when *carm1-/-* or *carm1-/-(+mut)* extracts were used (Fig.1B). Together these experiments confirmed that *carm1-/-* cells were complemented comparable to wild type cells.

A subset of TNFα-induced NF-κB-dependent genes required CARM1 for their expression.

Previously, we could show by conventional RT-PCR that some selected NF-κB dependent genes were differentially expressed in *carm1+/+* and *carm1-/-* cells. To have a broader view of which genes required CARM1 for their proper expression, global NF-κB-dependent gene expression was analyzed using a customized micro array with 350 sequences of previously identified or putative NF-κB target genes. Total RNA was isolated from *carm1-/-*, *carm1-/-(+wt)* or *carm1-/-(+mut)*, which were either untreated or stimulated for 3 hours with TNFα. RT-PCR for IκBα, a well known TNFα-dependent NF-κB target gene, revealed that IκBα expression was induced to the same extend in all three cell lines, thus confirming signal pathway activation by TNFα stimulation (data not shown). Amplified and labeled RNAs were subsequently co-hybridized in different combination to the customized micro array. First, we compared non-stimulated and TNFα-stimulated *carm1-/-(+wt)* cells. 28 genes were found to be up-regulated more than 2-fold upon stimulation (Fig.2A and 2B). No gene was found to be down-regulated upon TNFα stimulation. Comparison of the gene expression profile of TNFα-stimulated *carm1-/-* and *carm1-/-(+wt)* cells revealed that 26 genes were upregulated more than 2-fold in *carm1-/-(+wt)* compared to *carm1-/-* indicating that CARM1 is required for their expression (data not shown). From these 26 CARM1-dependent genes, only 7 genes were also dependent on TNFα stimulation (indicated in bold in Fig.2B) while the others were not induced by TNFα.

Next, we assessed the contribution of CARM1's enzymatic activity for the expression of NF-κB-dependent genes. Comparison of gene expression profiles of TNFα-stimulated *carm1-/-(+wt)* and *carm1-/-(+mut)* cells revealed that gene expression profiles were highly similar. Gene expression was subsequently confirmed for some genes by q-RT-PCR (TNFα-stimulated cells in Fig.3A and B and data not shown). Together these results provide strong evidence that CARM1 is

required for the expression of a subset of NF-κB target genes but the enzymatic activity of CARM1 is dispensable.

Induction of NF-κB by PMA/Ionomycin neither requires the enzymatic activity of CARM1.

The NF-κB response is in part dependent on the stimulus (33). To investigate whether CARM1 is also indispensable for the expression of a subset of NF-κB target genes when the complemented *carm1-/-* MEF cells are treated with another stimulus, experiments were repeated and cells stimulated for 3 hours either with TNFα or PMA/Ionomycin. IκBα and Cox2, two genes identified earlier in our studies as CARM1-independent genes (Fig.2), were induced upon TNFα stimulation in all three cell lines to about 8-fold or 5-fold, and upon PMA/Ionomycin stimulation to 2-fold or 20-fold, respectively, suggesting that cells were indeed stimulated by the treatment and confirmed CARM1 independency of these genes (Fig.3A).

We subsequently investigated the expression of four CARM1-dependent genes after TNFα stimulation by q-RT-PCR (Mpa2l, Cxcl5, IL6 or MIP2). While Mpa2l and Cxcl5 were induced in a CARM1 and TNFα-dependent manner (20-fold for Mpa2l and 4 fold for Cxcl5), PMA/Ionomycin treatment of the cells was not able to induce their expression (Fig.3B), thus not allowing the assessment of the influence of the PMA/Ionomycin stimulus. However, IL6 was induced 15-fold upon TNFα or PMA/Ionomycin stimulation in *carm1-/-(+wt)* cell line and MIP2 was induced by TNFα by 100-fold, while PMA/Ionomycin induced gene expression only by 20-fold (Fig.3B). Both genes were expressed at a very low level in *carm1-/-* cells upon stimulation with both stimuli, re-confirming that the expression was dependent on CARM1. Moreover, measurement of IL6 and MIP2 gene expression in *carm1-/-(+mut)* cells revealed that the expression levels were comparable to the ones measured in *carm1-/-(+wt)* cells, confirming that the methyltransferase activity is dispensable after TNFα but also after PMA/Ionomycin stimulation for both tested NF-κB dependent genes.

Together, these results indicate that the level of gene expression for a given gene is dependent on the stimulus, but that the dependency for CARM1 is not altered by the stimulation.

The enzymatic activity of CARM1 reduces MIP2 mRNA degradation.

The measured expression levels by qRT-PCR represent equilibrium of mRNA synthesis and degradation. CARM1 was shown to increase mRNA degradation of some specific mRNAs bearing AU-rich elements (ARE) at their 3'-untranslated regions (UTR) by methylating HuD (23). Many cytokines and chemokines mRNA harbors AREs at their 3'-UTR. Thus, we explored the possible role of CARM1 in the stability of two CARM1-dependent genes, MIP2 and Cxcl5. Cells were stimulated for 1 hour with TNFα before the addition of actinomycin D (Fig.4). Total RNAs were purified at different time points, reverse-transcribed and analyzed by qRT-PCR. Cxcl5 mRNA was really stable in the three different cell lines with a half-life of more than 2 hours (Fig.4A). However, MIP2 mRNA was less stable in the *carm1-/-(+wt)* cells (half-life of less than 30 minutes) compared to the stability in *carm1-/-* and *carm1-/-(+mut)* cells (half life of 1 hour, Fig.4B). These results indicate that although CARM1's enzymatic activity is not required for transcription, it has an effect in the MIP2 mRNA stability.

RelA/p65 recruitment to chromatin is stimulus-dependent but independent on CARM1.

To investigate whether CARM1 would regulate the recruitment of NF-κB to its target genes, we performed chromatin immunoprecipitation (ChIP) experiments of RelA/p65 to the promoter of NF-κB-dependent genes in *carm1-/-* cells, *carm1-/-(+wt)* and in *carm1-/-(+mut)* cells after TNFα stimulation. Recruitment to the promoter of CARM1-dependent (MIP2 and Mpa2l) and CARM1-independent genes (Cox2 and IP10) or control gene (glucagon) was analyzed by qPCR using specific primers. RelA/p65 was similarly recruited to promoters of CARM1-independent or dependent genes in all cell lines (Cox2, IP10, and Mpa2l, Fig.5A). Only at the MIP2 promoter in *carm1-/-* cells, RelA/p65 recruitment was delayed indicating that CARM1 protein might stabilize the binding of NF-κB to this gene. As a control, RelA/p65 was not recruited to the glucagon promoter (Fig.S1).

Wild type and enzymatically inactive CARM1 are recruited to the same extent to NF-κB target genes.

To control that both complemented forms of CARM1 would be recruited to comparable levels, we repeated ChIP experiments for CARM1 and the indicated promoters (Fig.5B and Fig.S1). Wild type and mutant CARM1 were recruited to the same extent after 30 minutes of stimulation to all tested promoters. The recruitment would increase over time (highest at 60 minutes) for wild type CARM1 and slightly decreases, although not significantly, at the later time point for mutant CARM1 (Fig.5B). As a control, CARM1 was not recruited to the glucagon promoter (Fig.S1).

DISCUSSION

To further characterize CARM1 as a transcriptional coactivator, *carm1-/-* cells were stably complemented by viral transduction with wild type or with an enzymatic inactive mutant of CARM1. Cells were subsequently stimulated by TNFα or PMA/Ionomycin and gene expression analyzed using either a custom micro array with NF-κB-target genes or by qRT-PCR. Both analyses reveal that CARM1 is required for the correct expression of certain NF-κB-target genes but that the enzymatic activity of CARM1 is dispensable. The absence of the enzymatic activity did neither affect the recruitment of RelA/p65 nor the one of CARM1 to distinct promoters. However, the enzymatic activity affected the mRNA stability of MIP2.

So far, functional contribution of CARM1 upon induced gene expression was never analyzed on a global level. Here, CARM1's functional role as a transcriptional coactivator was analyzed in more details using a customized micro array for NF-κB target genes. This global approach revealed that only a subset of genes required the presence of the protein (independently of its enzymatic activity) probably dependent on the promoter context. Furthermore, few studies analyzed the *in vivo* expression of certains genes by RT-PCR in knockout or knockdown cells (14,15,27,34,35). Thus, further studies will be necessary to extend the view that CARM1 is necessary only for a subset of target genes and to define which promoter context requires CARM1. Additionally, we found that complementation of *carm1-/-* cells with wt or inactive CARM1 changed the expression profile in part independent on TNFα (Fig.2 and data not shown), indicating that CARM1 might influence the composition of the chromatin on a general level.

Interestingly, CARM1 was not required for the recruitment of NF-κB upon TNFα stimulation, since NF-κB was recruited after 30 and 60 min to three different promoters (Cox2, MPa2l and IP10) in all tested cells lines in a stimuli-dependent manner to the same extent and only slightly delayed to the MIP2 promoter. These results suggests, that while CARM1 is influencing the initial recruitment of NF-κB to its response elements (11), CARM1 does not influence the chromatin binding of NF-κB upon longer stimulation with TNFα. Interestingly, the binding of NF-κB itself to these promoters was not sufficient to induce gene expression in *carm1-/-* cells (Fig.3B). Furthermore, CARM1 was recruited in a stimuli-dependent manner, independent whether it was an active or inactive protein, suggesting that CARM1 is important for transcriptional coactivator complex formation and/or stabilization of this complex.

Comparable to TNFα, also PMA/Ionomycin induced NF-κB-dependent gene expression was not dependent on CARM1's enzymatic activity, suggesting that the observed dispensability might be generally valid. We can however, not exclude, that other stimuli or cell types might depend on the enzymatic activity of CARM1 and that also the promoter context might influence the requirement of protein methylation by CARM1. Further studies are needed to extend our observations.

Despite intensive investigation regarding the involvement of CARM1's enzymatic activity for its role as transcriptional coactivator, its contribution is still not clear and even controversial. Methylation of p300 by CARM1 could be beneficial (17) or could attenuate transcriptional responses (18). On the other hand, methylation of SRC3/AIB1/p/CIP attenuated estrogen receptor transcriptional response by destabilizing complex association (19,24). This is the first report providing genetical evidences that the enzymatic activity is not required for the transcriptional activation of genes. The contribution of CARM1's enzymatic activity was so far investigated by comparing gene expression profiles from *carm1-/-* and *carm1+/+* cells, which does not allow deciphering between requirement

of the protein or its enzymatic activity (11,15,26,27). Additionally, the requirement of CARM1 enzymatic activity to coactivate gene expression was mainly analyzed by overexpressing CARM1 and using transiently transfected reporter gene expression assay as readout (13,31). This latter approach, however, suffers major drawbacks such as the transfection of multiple plasmids, which are poorly chromatinized and the overexpression of proteins that could impair the balance of proteins in the cell. Consequently, the results of such experiment might not reflect the *in vivo* functional relevance of CARM1. In the context of multiple protein-protein complexes present at the promoters during the initiation step of transcription, the requisite of CARM1 protein might not be compensated by other proteins. CARM1 possesses a unique C-terminal extension that bears an autonomous activation domain (36) which suggests that CARM1 may interact with downstream components of the transcriptional machinery. Since the presence of CARM1 is important to induce a proper expression of NF-κB-dependent genes, it might be through the recruitment of other important coactivator or stabilization of complexes. Few proteins are known to interact with full-length CARM1 such as Brg1 within the NUMAC complex (37), p300 (8,11,18), SRCAP, which shares homology with the SNF2 family of proteins (38) or β-catenin (39). A recent study revealed that CARM1 could facilitate the recruitment of Brg1 at late genes during muscle differentiation (40). Interestingly, not only wild-type but also the enzymatic mutant of CARM1 was reported to synergistically activate together with Brg1 ER-dependent transcription (37). Further studies are needed to identify and characterize the proteins that are dependent on CARM1 and that participate in the appropriate expression of NF-κB-dependent genes.

Moreover, besides its role as a transcriptional coactivator, CARM1 was shown to affect mRNA stability/degradation of some specific mRNAs bearing AU-rich elements (ARE) at their 3'-untranslated regions (UTR) by methylating HuD (23). Many cytokines and chemokines mRNA harbors AREs at their 3'-UTR. Experiments of gene expression in presence of actinomycin D indeed revealed that MIP2 mRNA was less stable in the *carm1-/-(+wt)* cell line (Fig.4). Thus, the enzymatic activity of CARM1 might not be required for transcriptional coactivation, but might rather influence mRNA stability. mRNA stability was recently reported to be a key determinant controlling the kinetic patterns of TNFα-induced gene expression (41).

Overexpression of CARM1 was observed in hormone-independent prostate carcinoma, suggesting that overexpressed CARM1 could lead to tumor formation by stabilizing coactivator complexes at the androgen receptor target genes (42,43). The contribution of the enzymatic activity has to be further investigated, but based on our observation the overexpression of CARM1 alone might be sufficient to tumorigenesis.

Together, the presented results strongly support an essential role of the CARM1 as transcriptional coactivator. Moreover, our results suggest that the enzymatic activity of CARM1 is dispensable for NF-κB-dependent gene expression.

REFERENCES

1. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu Rev Immunol* **16**, 225-260
2. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) *Nat Rev Cancer* **2**, 301-310
3. Bonizzi, G., and Karin, M. (2004) *Trends Immunol* **25**, 280-288
4. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) *Genes Dev* **20**, 1405-1428
5. Lonard, D. M., and O'Malley B, W. (2007) *Mol Cell* **27**, 691-700
6. Taverna, S. D., Li, H., Ruthenburg, A. J., Allis, C. D., and Patel, D. J. (2007) *Nat Struct Mol Biol* **14**, 1025-1040
7. Seet, B. T., Dikic, I., Zhou, M. M., and Pawson, T. (2006) *Nat Rev Mol Cell Biol* **7**, 473-483
8. Hassa, P. O., Covic, M., Bedford, M. T., and Hottiger, M. O. (2008) *J Mol Biol* **377**, 668-678
9. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc Natl Acad Sci U S A* **94**, 2927-2932

10. Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999) *Mol Cell Biol* **19**, 6367-6378
11. Covic, M., Hassa, P. O., Saccani, S., Buerki, C., Meier, N. I., Lombardi, C., Imhof, R., Bedford, M. T., Natoli, G., and Hottiger, M. O. (2005) *EMBO J* **24**, 85-96
12. Lee, D. Y., Teyssier, C., Strahl, B. D., and Stallcup, M. R. (2005) *Endocr Rev* **26**, 147-170
13. Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999) *Science* **284**, 2174-2177
14. Kleinschmidt, M. A., Streubel, G., Samans, B., Krause, M., and Bauer, U. M. (2008) *Nucleic Acids Res* **36**, 3202-3213
15. Fauquier, L., Duboe, C., Jore, C., Trouche, D., and Vandel, L. (2008) *FASEB J* **22**, 3337-3347
16. Bauer, U. M., Daujat, S., Nielsen, S. J., Nightingale, K., and Kouzarides, T. (2002) *EMBO Rep* **3**, 39-44
17. Chevillard-Briet, M., Trouche, D., and Vandel, L. (2002) *EMBO J* **21**, 5457-5466
18. Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B. M., Montminy, M., and Evans, R. M. (2001) *Science* **294**, 2507-2511
19. Feng, Q., Yi, P., Wong, J., and O'Malley, B. W. (2006) *Mol Cell Biol* **26**, 7846-7857
20. Lee, Y. H., Coonrod, S. A., Kraus, W. L., Jelinek, M. A., and Stallcup, M. R. (2005) *Proc Natl Acad Sci U S A* **102**, 3611-3616
21. Lee, J., and Bedford, M. T. (2002) *EMBO Rep* **3**, 268-273
22. Li, H., Park, S., Kilburn, B., Jelinek, M. A., Henschen-Edman, A., Aswad, D. W., Stallcup, M. R., and Laird-Offringa, I. A. (2002) *J Biol Chem* **277**, 44623-44630
23. Fujiwara, T., Mori, Y., Chu, D. L., Koyama, Y., Miyata, S., Tanaka, H., Yachi, K., Kubo, T., Yoshikawa, H., and Tohyama, M. (2006) *Mol Cell Biol* **26**, 2273-2285
24. Naeem, H., Cheng, D., Zhao, Q., Underhill, C., Tini, M., Bedford, M. T., and Torchia, J. (2007) *Mol Cell Biol* **27**, 120-134
25. Torres-Padilla, M. E., Parfitt, D. E., Kouzarides, T., and Zernicka-Goetz, M. (2007) *Nature* **445**, 214-218
26. Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M. C., Aldaz, C. M., and Bedford, M. T. (2003) *Proc Natl Acad Sci U S A* **100**, 6464-6468
27. Yadav, N., Cheng, D., Richard, S., Morel, M., Iyer, V. R., Aldaz, C. M., and Bedford, M. T. (2008) *EMBO Rep* **9**, 193-198
28. Troffer-Charlier, N., Cura, V., Hassenboehler, P., Moras, D., and Cavarelli, J. (2007) *EMBO J* **26**, 4391-4401
29. Yue, W. W., Hassler, M., Roe, S. M., Thompson-Vale, V., and Pearl, L. H. (2007) *EMBO J* **26**, 4402-4412
30. El-Andaloussi, N., Valovka, T., Toueille, M., Steinacher, R., Focke, F., Gehrig, P., Covic, M., Hassa, P. O., Schar, P., Hubscher, U., and Hottiger, M. O. (2006) *Mol Cell* **22**, 51-62
31. Lee, Y. H., Koh, S. S., Zhang, X., Cheng, X., and Stallcup, M. R. (2002) *Mol Cell Biol* **22**, 3621-3632
32. Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002) *Nucleic Acids Res* **30**, e36
33. Hoffmann, A., and Baltimore, D. (2006) *Immunol Rev* **210**, 171-186
34. El Messaoudi, S., Fabbrizio, E., Rodriguez, C., Chuchana, P., Fauquier, L., Cheng, D., Theillet, C., Vandel, L., Bedford, M. T., and Sardet, C. (2006) *Proc Natl Acad Sci U S A* **103**, 13351-13356
35. Frietze, S., Lupien, M., Silver, P. A., and Brown, M. (2008) *Cancer Res* **68**, 301-306
36. Teyssier, C., Chen, D., and Stallcup, M. R. (2002) *J Biol Chem* **277**, 46066-46072
37. Xu, W., Cho, H., Kadam, S., Banayo, E. M., Anderson, S., Yates, J. R., 3rd, Emerson, B. M., and Evans, R. M. (2004) *Genes Dev* **18**, 144-156
38. Monroy, M. A., Schott, N. M., Cox, L., Chen, J. D., Ruh, M., and Chrivia, J. C. (2003) *Mol Endocrinol* **17**, 2519-2528

39. Koh, S. S., Li, H., Lee, Y. H., Widelitz, R. B., Chuong, C. M., and Stallcup, M. R. (2002) *J Biol Chem* **277**, 26031-26035
40. Dacwag, C. S., Bedford, M. T., Sif, S., and Imbalzano, A. N. (2009) *Mol Cell Biol*
41. Hao, S., and Baltimore, D. (2009) *Nat Immunol* **10**, 281-288
42. Hong, H., Kao, C., Jeng, M. H., Eble, J. N., Koch, M. O., Gardner, T. A., Zhang, S., Li, L., Pan, C. X., Hu, Z., MacLennan, G. T., and Cheng, L. (2004) *Cancer* **101**, 83-89
43. Majumder, S., Liu, Y., Ford, O. H., 3rd, Mohler, J. L., and Whang, Y. E. (2006) *Prostate* **66**, 1292-1301

FOOTNOTES

We are grateful to Dr. Mark T. Bedford (The University of Texas M.D. Anderson Cancer Center, Science Park –Research Division, Smithville, Texas), Dr. Michael Grunstein (University of California, Los Angeles) and Dr. Gioacchino Natoli (IFOM-IEO, Italy) for the generous provision of reagents and sequences. Dr. Taras Valovka and the Functional Genomics Center Zürich (Switzerland) are acknowledged for their technical support. This work was supported in part by the Novartis Foundation and the Bonizzi-Theler Foundation (to S.J.), by Swiss National Science Foundation Grant 31-109315 and 31-122421 (to K.R.) and the Kanton of Zurich (to M.O.H.).

FIGURE LEGENDS

Fig. 1. Genetic complementation of *carm1-/-* cells. *A* Whole cell extracts of the different cell lines were analyzed by western blot using anti-CARM1 and anti-PRMT1 antibodies, *B* purified GST-PABP was incubated with ^{14}C-SAM and whole cell extracts from *carm1-/-*, *carm1-/-(+wt)* and *carm1-/-(+mut)* cells as a source of CARM1 or purified recombinant CARM1. Pull-down GST-PABP was loaded on a SDS-PAGE and analyzed by autoradiography.

Fig. 2. CARM1 is required for the expression of a subset of NF-κB-dependent genes. *A* Venn diagram showing upregulated genes found in the micro array analysis when non-stimulated *carm1-/-(+wt)* cells and TNFα-stimulated *carm1-/-* cells were compared to TNFα-stimulated *carm1-/-(+wt)* cells. *B* List of genes found in the micro array analysis. CARM1-dependent genes are indicated in bold. The fold induction after TNFα stimulation represents the increase in gene expression obtained when comparing non-stimulated and TNFα-stimulated *carm1-/-(+wt)* cells. CARM1 dependency was determined by comparing TNFα-stimulated *carm1-/-(+wt)* and *carm1-/-* cells.

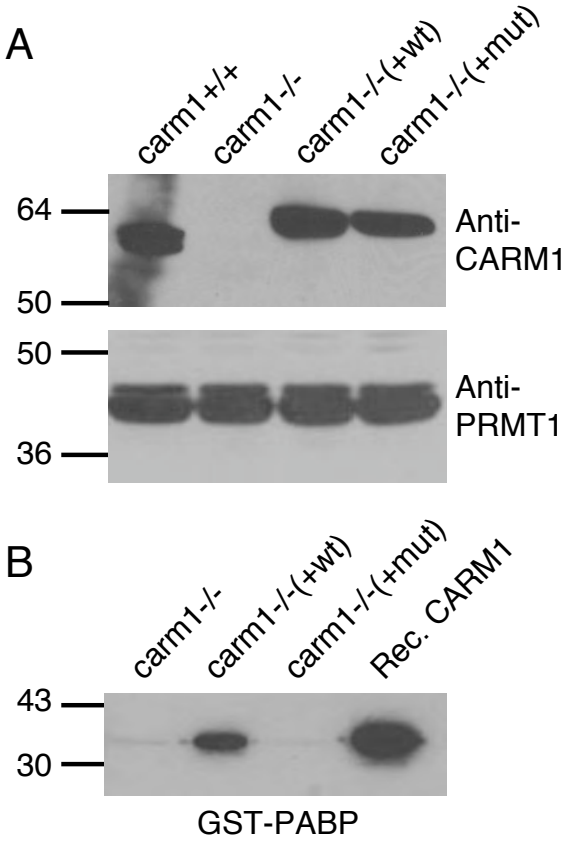
Fig. 3. Enzymatic activity of CARM1 is not required for the expression of NF-κB target genes. *Carm1-/-*, *carm1-/-(+wt)* and *carm1-/-(+mut)* cells were left unstimulated (white bars), stimulated with TNFα (black bars) or with PMA/Ionomycine (grey bars). *A* The relative mRNA expression of two CARM1-independent genes (IκBα and Cox2) and *B* four CARM1-dependent genes (IL6, MIP2, Mpa2l and Cxcl5) are shown. Results are representative of three biological replicates.

Fig. 4. MIP2 mRNA stability is influenced by CARM1's enzymatic activity. Cells were stimulated for one hour with TNFα (time 0) and mRNA synthesis was inhibited by actinomycin D for different time points. *A* Expression level of remaining MIP2 and *B* Cxcl5 in *carm1-/-* (◆), *carm1-/-(+wt)* (■) and *carm1-/-(+mut)* (▲) mRNAs was determined and calculated relative to time 0 (set at 100%).

Fig. 5. p65 recruitment is not impaired in *carm1-/-* cells. Fold increase in the recruitment of *A* RelA/p65 and *B* Flag-CARM1 at promoters of two CARM1-dependent genes (MIP2 and Mpa2l) and of two

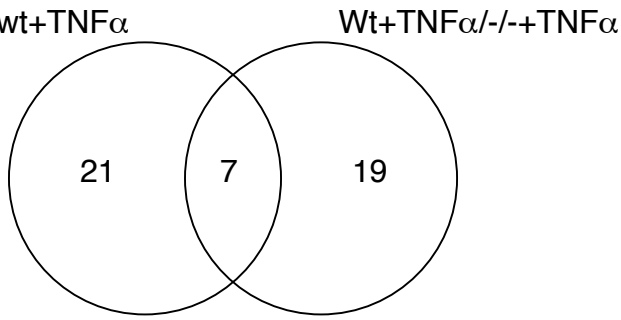
CARM1-independent genes (Cox2 and IP10) was analyzed by ChIP experiments in non-treated cells (white bars), after 30' (black bars) or 60' (grey bars) stimulation with TNFα.

Jayne et al., Figure 1



Jayne et al., Figure 2

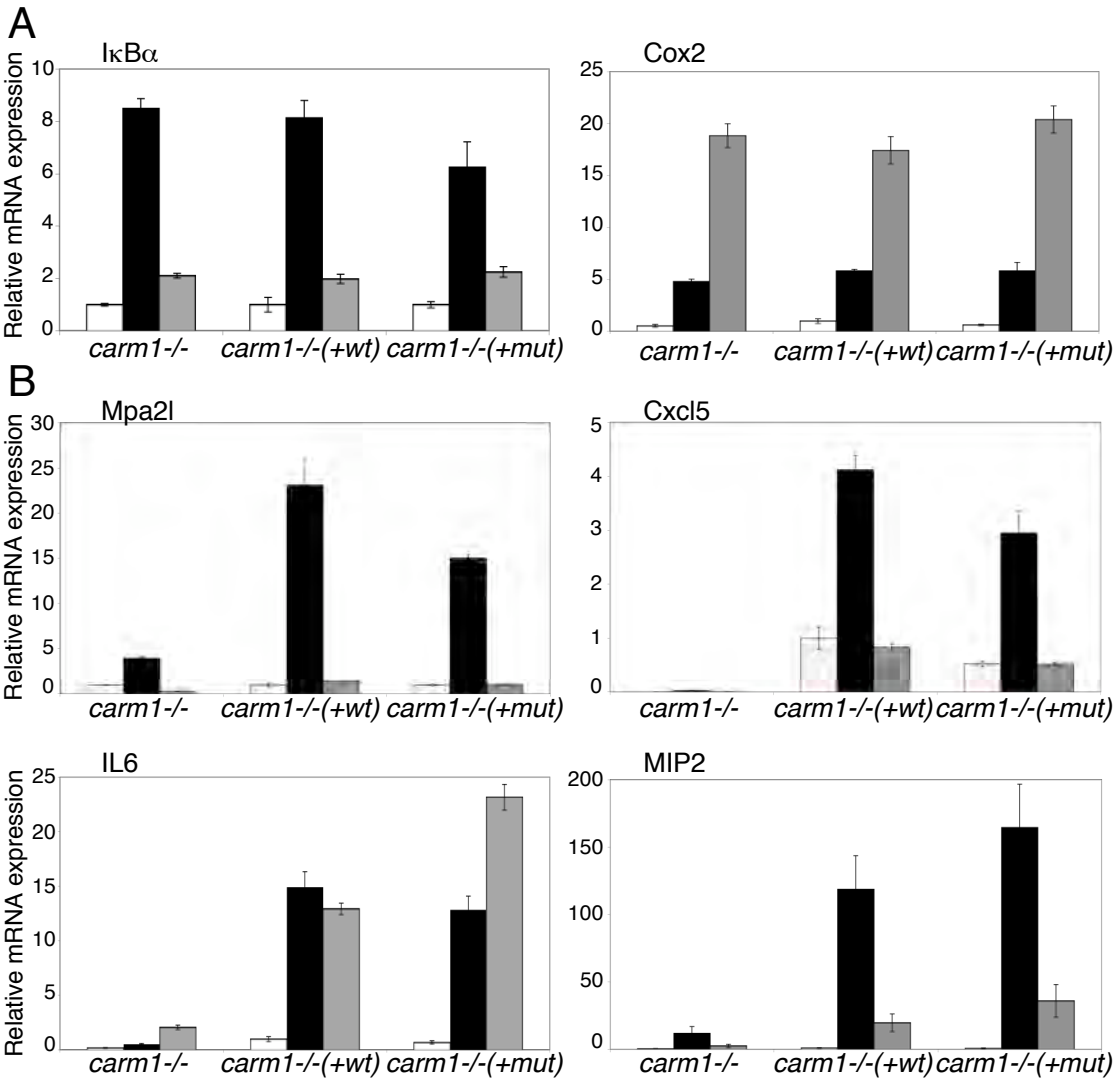
A



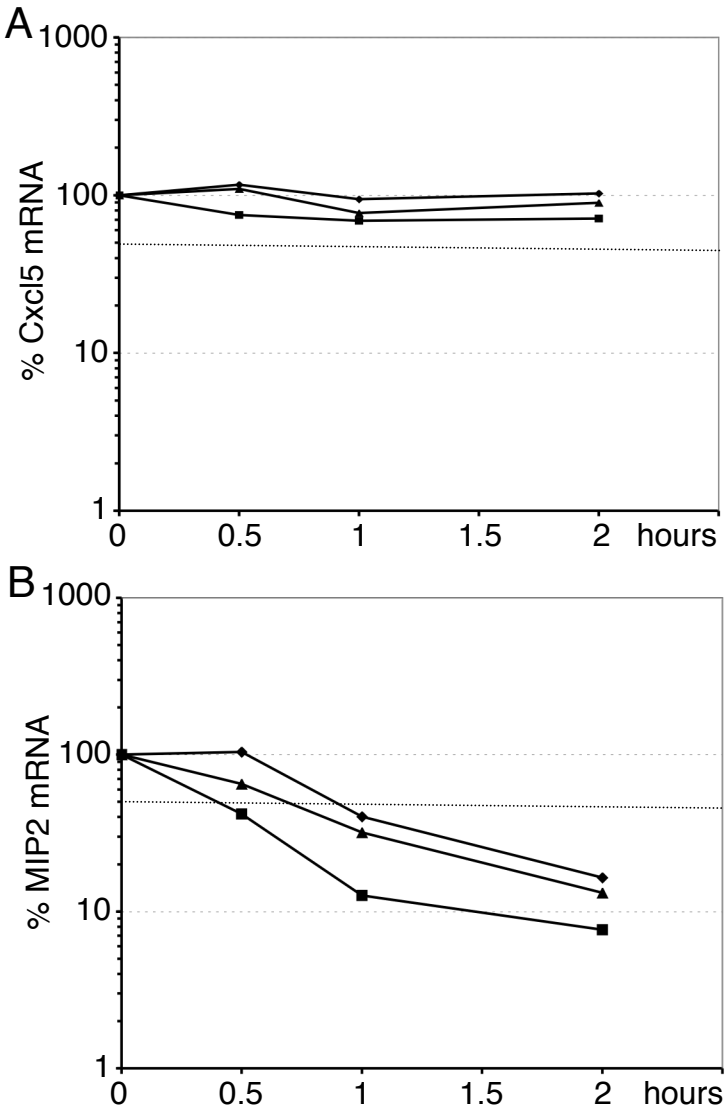
B

Genbank Accession number	Gene common name	Fold induction after TNFα stimulation	CARM1 dependency
NM_013671	Sod2	2.01	
NM_011905	Tir2	2.06	
NM_013653	Ccl5	2.081	
NM_008194	Gyk	2.122	
NM_007987	Tnfrsf6	2.126	
NM_007670	Cdkn2b	2.137	
NM_007778	Csf1	2.198	
NM_009046	Relb	2.232	
NM_009141	**Cxcl5**	2.441	7.881
NM_008416	Junb	2.454	
NM_011333	Ccl2	2.476	
NM_009969	**Csf2**	2.567	2.836
NM_007464	Birc3	2.588	
NM_009140	**Cxcl2/MIP2**	2.877	3.072
NM_011198	Ptgs2/Cox2	2.904	
NM_009137	Ccl22	2.913	
NM_007628	Ccna1	3.228	
NM_011693	**Vcam1**	3.451	2.183
NM_013654	Ccl7	3.455	
NM_009915	Ccr2	3.824	
NM_021274	Cxcl10/IP10	4.01	
NM_008690	Nfkbie	4.062	
NM_010907	Nfkbia	4.145	
NM_009397	Tnfaip3	4.992	
NM_031168	**Il6**	5.132	11.235
NM_145700	**Ccrl1**	5.211	2.016
NM_194336	**Mpa2l**	5.635	6.003
NM_016960	Ccl20	6.716	

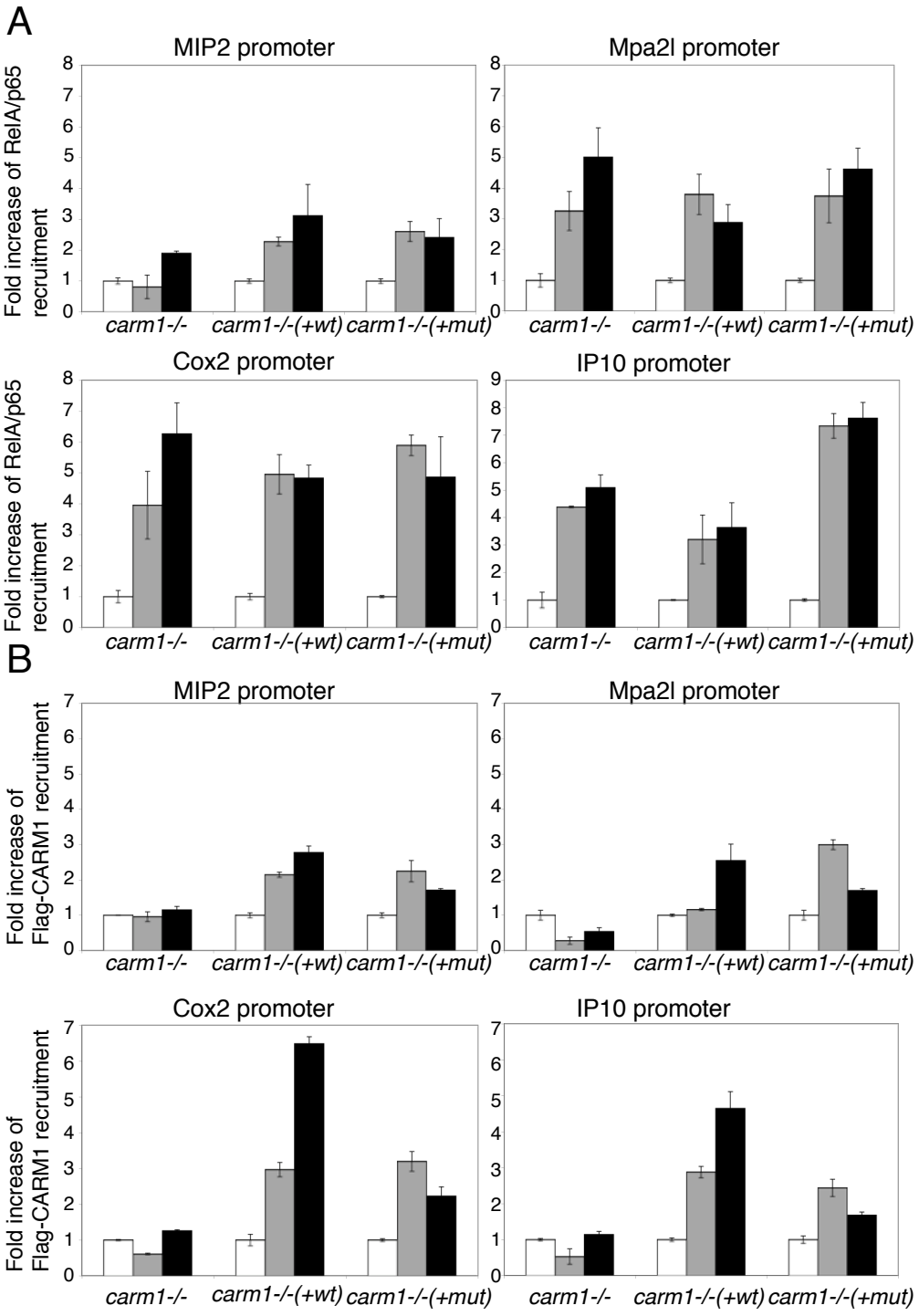
Jayne et al., Figure 3



Jayne et al., Figure 4



Jayne et al., Figure 5



Supplementary Tables

Table1: Sequences of the oligonucleotides used for gene expression and for ChIP analysis

Oligonucleotides used for gene expression analysis		
	Primer	Primer Sequences
Rps12	Fw	GAAGCTGCCAAAGCCTTAGA
	Rev	AACTGCAACCAACCACCTTC
Cox2	Fw	AGGGCCCTACCAAGATGCTAGAAA
	Rev	CCCTATTGCCCAGAACTACTCACA
IκBα	Fw	AAATCTCCAGATGCTACCCGAGAG
	Rev	ATAATGTCAGACGCTGGCCTCCAA
IL6	Fw	TCACTTTGAGATCTACTCGGCAAACC
	Rev	TCTGACCACAGTGAGGAATGTCCA
MIP2	Fw	ACATCCCACCCACACAGTGAAAGA
	Rev	TCCTTCCATGAAAGCCATCCGACT
Cxcl5	Fw	GCTGCTGTGTCATGCAGAAACCTA
	Rev	GACATTATGCCATACTACGAAGACATC
Mpa2l	Fw	CTTGGAGAAGCCTACTTCGTCTCT
	Rev	AAATCTGCCAGCAGACCCTAACCT

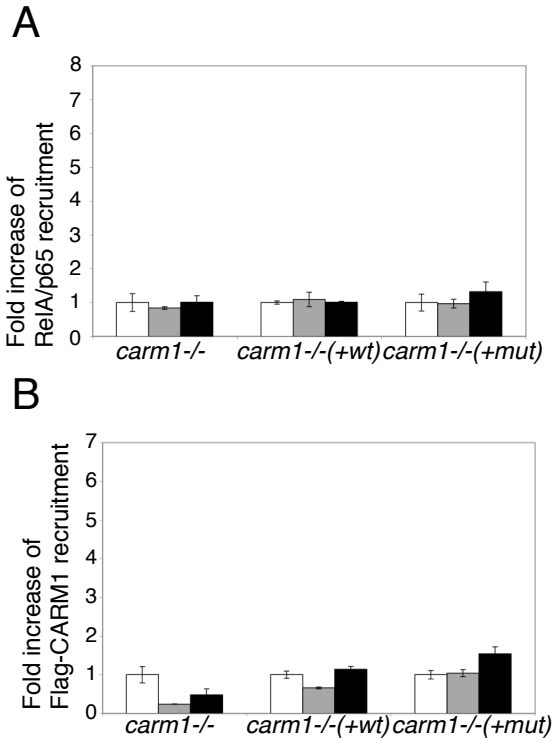
Oligonucleotides used for ChIP analysis		
	Primer	Primer Sequences
IP10	Fw	GCAATGCCCTCGGTTTACAG
	Rev	GGCTGACTTTGGAGATGACTCA
Cox2	Fw	AGTACAGATGTGGACCCTGACAGAG
	Rev	GGCCTAAAAGCTCAAGAGTGTCACAG
Mpa2l	Fw	CAGCCCCTTTTATAGTGAGTC
	Rev	TACAAAATCCGGGAGTATTGC
MIP2	Fw	CCCAGGGTCCCATAGTGGAA
	Rev	CCGCCCCTAAGCTGAGT
Glucagon	Fw	GAGTGGGCGAGTGAAATCAT
	Rev	TGAGCTGCGAACAGGTGTAG

Supplementary Figure Legend

Fig.S1 RelA/p65 and Flag-CARM1 are not recruited at the glucagon promoter

Fold increase in the recruitment of RelA/p65 (*A*) and Flag-CARM1 (*B*) at the promoter of glucagon gene was analyzed by ChIP experiments in non-treated cells (white bars), after 30' (black bars) or 60' (grey bars) stimulation with TNFα.

Jayne et al., Figure S1



7 Unpublished data

7.1 Acetylation of p65 in response to TNFα and IL-1β stimulation

Proinflammatory cytokines such as TNFα and IL-1β trigger the classical NF-κB pathway [266]. The finding that p65 is acetylated after TNFα stimulation prompted us to test whether this was also the case after IL-1β treatment. Indeed, p65 acetylation can be observed in *p65*$^{-/-}$ MEFs complemented with p65 wild type after stimulation with IL-1β, as revealed by western blot using the specific antibody against acetylated K310 from p65 (Figure 8). The observed signal is stronger than the one after TNFα stimulation. Together, this data suggest that p65 acetylation is in general important for gene expression.

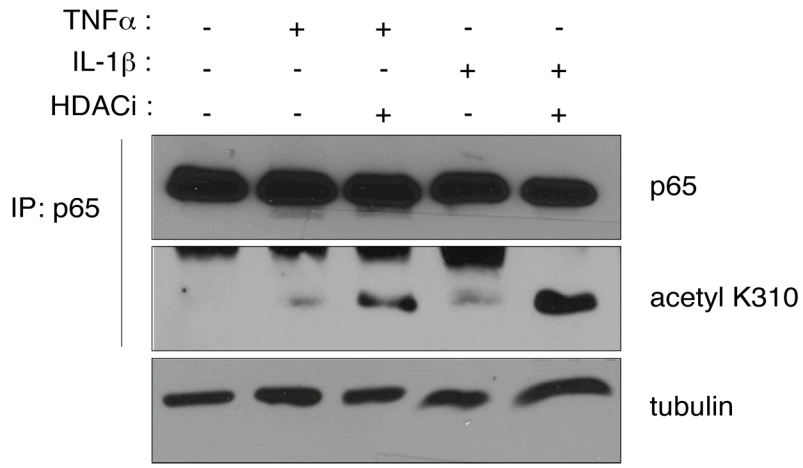


Figure 8. *Endogenous p65 acetylation at K310 upon TNFα and IL-1β stimulation in vivo.* Wild type complemented MEFs were treated with or without HDAC inhibitors (HDACi) TSA and Nam for 30 minutes before stimulation with TNFα or IL-1β for 45 minutes. Total cell extracts were prepared, p65 was immunoprecipitated and samples were analyzed by western blot using α-acetyl K310 antibody. The membrane was reprobed for p65. Tubulin from 5% extracts was used as loading control.

7.2 Characterization of α-acetyl K314/315 p65 specific antibody

Since we were able to detect acetylation at K314 and K315 *in vitro* and when p65 and p300 were overexpressed in HEK 293T cells, but not with endogenous proteins with the specific antibodies against acetylated K314 or acetylated K315, we decided to generate an antibody that recognizes both acetylated lysines. This antibody was generated by Eurogentec using the following peptide for immunization: SIM K(Ac)K(Ac)S PFS GPC, acetylated at both lysines.

Recombinant p65 protein was acetylated *in vitro* by p300 and analyzed by western blot with purified α-acetyl K314/315 antibody (Figure 9A). To determine if the signal was specific, p65 K314/315R mutant protein was used as a negative control. The antibody detected acetylated p65 only when p65 wild type protein was used. In addition, the antibody specifically recognized acetylated p65 overexpressed in HEK 293T cells together with p300 (Figure 9B). However, the antibody failed to detect endogenous acetylated p65 from nuclear or total cell extracts (Figure 9C and 9D, respectively). In both experiments, no band from the size of p65 was observed, although complemented cells were treated with HDACi to facilitate the detection of endogenous acetylated p65, as has been done for detection of acetylated K310.

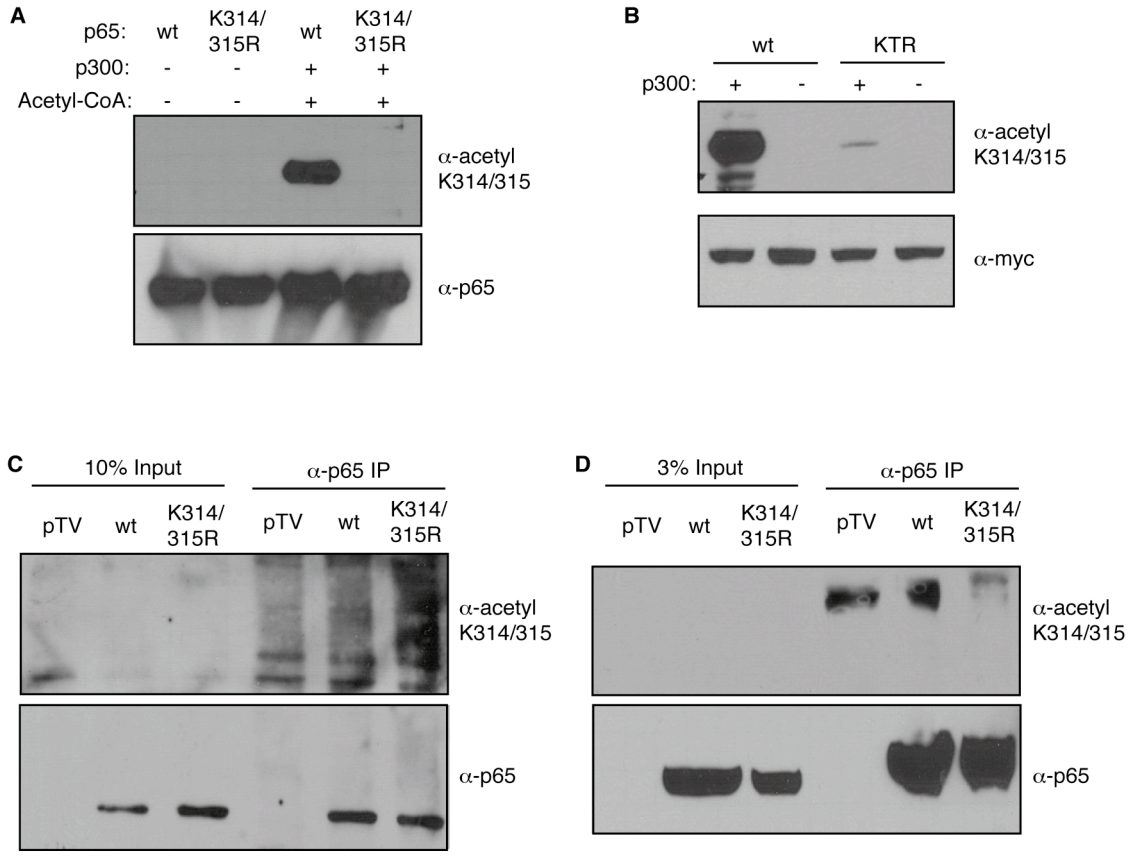


Figure 9. *Characterization of purified α-acetyl K314/315 antibody*. **(A)** Recombinant purified p65 wild type and K314/315R mutant were acetylated *in vitro* by p300 and analyzed by western blot with α-acetyl K314/315 antibody. The membrane was reprobed with α-p65 antibody. **(B)** Myc-tagged p65 wild type and p65 KTR mutant were overexpressed in HEK 293T cells with or without co-expression of p300. Total cell extracts were prepared and analyzed by western blot with the indicated antibodies. **(C)** Complemented cells were treated with TSA and Nam for 1 hour prior TNFα stimulation for 20 minutes. Nuclear extracts were prepared, p65 was immunoprecipitated and analyzed by western blot. **(D)** Complemented cells were treated with TSA and Nam for 2.5 hours before stimulation with TNFα for 30 minutes. p65 was immunoprecipitated from total extracts and analyzed by western blot with α-acetyl K314/315 antibody. The membrane was reblotted with α-p65 antibody.

Furthermore, the α-acetyl K314/315 serum was tested for the recognition of endogenous p65 in its native form. Immunostaining with the α-acetyl K314/315 serum in the wild type complemented cells showed a different staining pattern than the one obtained with the α-p65 antibody (Figure 10). The cross-reactivity of the antibody was further confirmed when pTV cells were used (*p65*$^{-/-}$ complemented with the empty vector), since the same unspecific signal was observed in both wild type and pTV cells. This result suggests that the α-acetyl K314/315 serum recognizes a different nuclear protein than p65, which is acetylated, since the signal was increased after treatment with HDACi. This serum might thus be used to study a perhaps so far unidentified acetylated protein in MEFs.

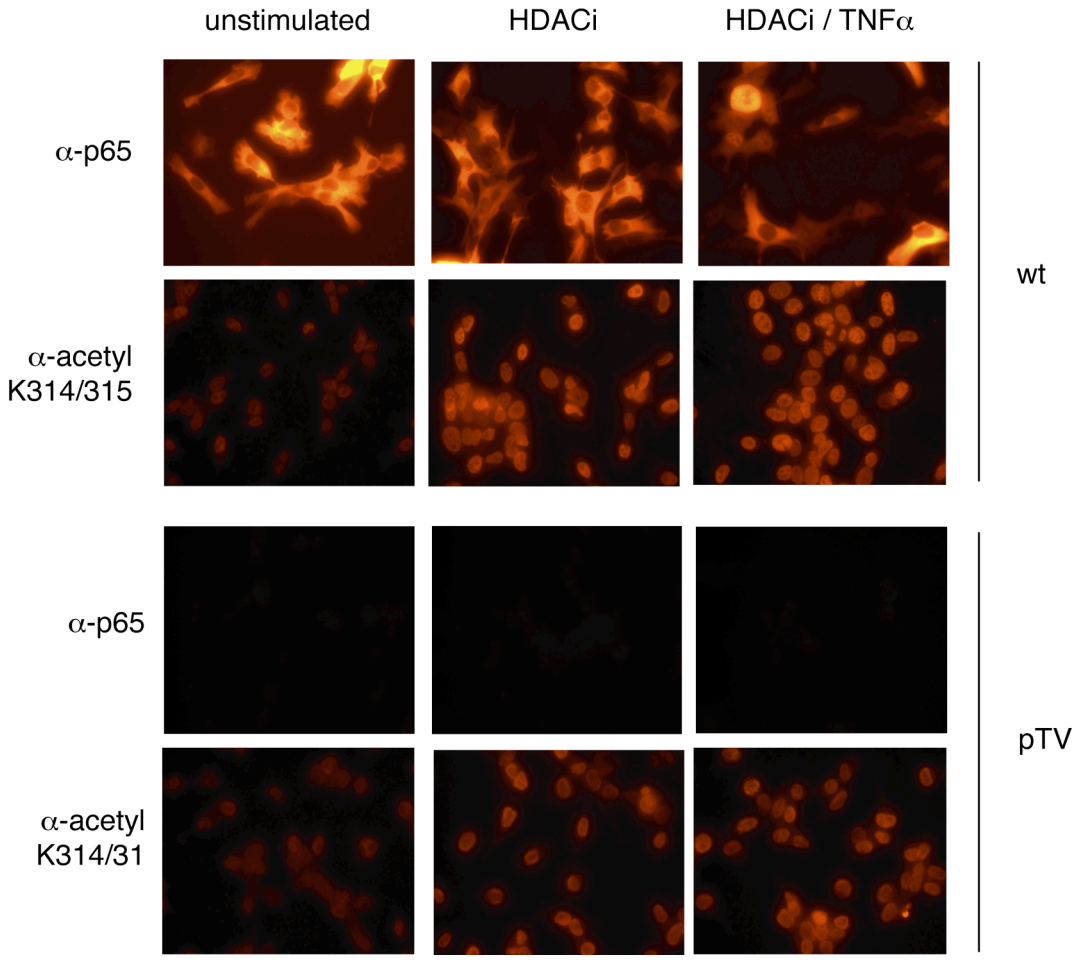


Figure 10. *Immunostaining using the α-acetyl K314/315 serum. p65*$^{-/-}$ MEFs complemented with p65 wild type (upper panel) or with the empty vector (lower panel) were either left untreated, treated with HDACi alone, or with both HDACi and TNFα. Cells were fixed and immunostained using α-acetyl K314/315 serum or α-p65 antibody.

7.3 Searching for acetylation-dependent interacting partners of p65

In order to modulate the transcription of target genes, p65 interacts with distinct coactivators and corepressor. In some cases, the interaction has been shown to depend on posttranslational modifications, such as the requirement of p65 phosphorylation at serines 276 and 536 for the binding with p300/CBP [260]. To determine whether p65 acetylation regulates NF-κB-dependent gene expression by modulating the ability of p65 to interact with specific coregulatory proteins, we performed several acetylation-dependent interaction studies. We focused on bromodomain-containing proteins, since bromodomains have been reported to bind acetyl lysines and are found in chromatin associated proteins and HATs [114].

7.3.1 p300

The bromodomain of p300 has been reported to bind to acetylated proteins including p53 and MyoD [267, 268]. To determine if p300 preferentially binds to acetylated p65 compared to non-acetylated one, we performed pull-down experiments using *in vitro* translated p300 bromodomain and GST-p65 wild type or KTR mutant. In contrast to what we expected, we observed an increased interaction with the non-acetylated p65 wild type or KTR mutant (Figure 11), suggesting that acetylation of p65 is not important for binding to p300.

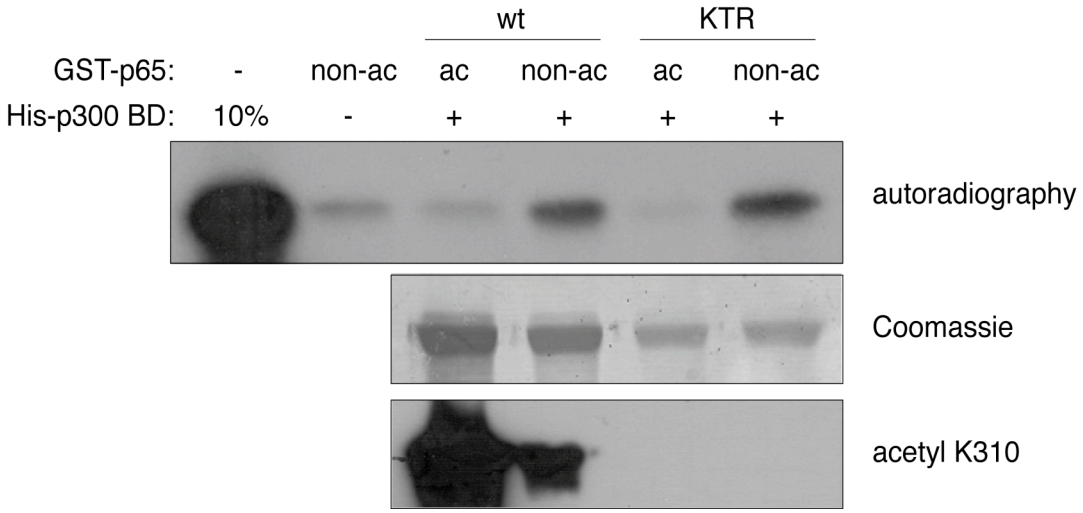


Figure 11. *In vitro interaction between GST-p65 and His-p300 BD*. GST-p65 was acetylated *in vitro* by p300 (ac) or left non-acetylated (non-ac). Pull-down assays were performed with radioactively labeled His-p300 bromodomain (His-p300 BD) and detected by autoradiography. 10% of His-p300 BD used for the pull-downs was kept as control. GST-p65 wild type or KTR mutant were either stained with coomassie as loading control or analyzed by western blot using α-acetyl K310 antibody.

7.3.2 TAF1

TAF1, the largest TAF of TFIID, contains two bromodomains that have been shown to interact with acetylated Histone H4 and p53 [95, 269]. Pull-down experiments between GST-TAF1 double bromodomain (GST-TAF1 BD) and recombinant p65 wild type revealed that these two proteins interact *in vitro* in an acetylation-dependent manner (Figure 12, lower panel). However, when p65 acetylation-deficient mutants were used for this experiment, we did not observe any decrease in the binding to TAF1 double bromodomain, suggesting that this interaction is not mediated by acetylation on lysines 218, 221, 310, 314 or 315 from p65.

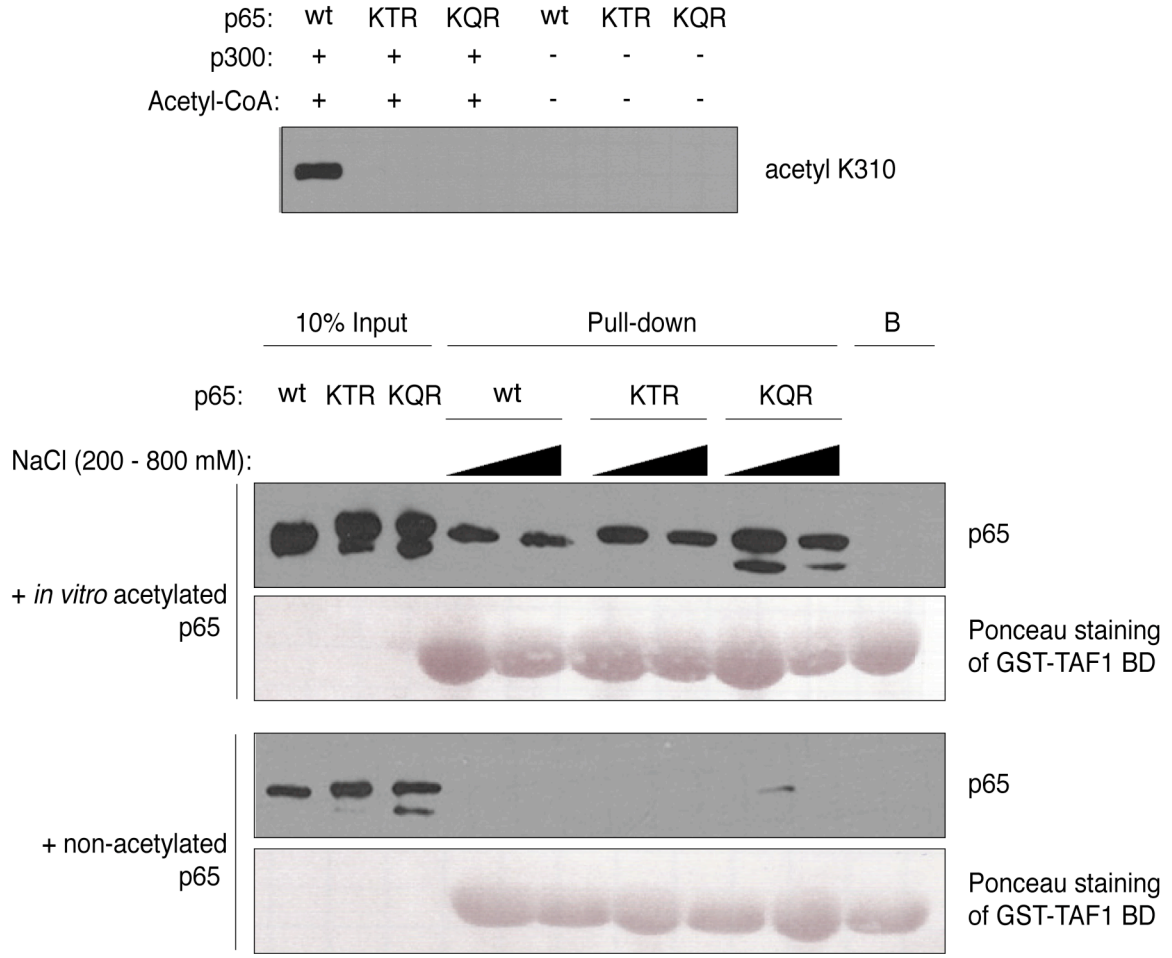


Figure 12. *Pull-down assays between TAF1 double bromodomain and purified p65*. Recombinant p65 wild type (wt) or acetylation deficient mutants KTR (K310/314/315R) or KQR (K218/221/310/314/315R) were acetylated *in vitro* by p300 in the presence of acetyl-CoA, as revealed by western blot analysis using α-acetyl K310 antibody or left non-acetylated (upper panel). Recombinant p65 proteins (wt or mutants, acetylated or non-acetylated) were incubated with GST-tagged TAF1 double bromodomain bound to beads (lower panel). Samples were washed with binding buffer containing 200 or 800 mM NaCl and analyzed by western blot using α-p65 antibody. Membranes were stained with Ponceau to detect GST-TAF1 BD. 10% input of recombinant p65 proteins were kept as positive control, GST-TAF1 BD bound to beads (B) alone was used as negative control.

To confirm that the binding between p65 and TAF1 depends on the bromodomains of TAF1, pull-down experiments with wild type or mutant double bromodomain were performed (Figure 13). Interaction was lost when the bromodomains were mutated, indicating that the integrity of the double bromodomain of TAF1 is required for its interaction with acetylated p65.

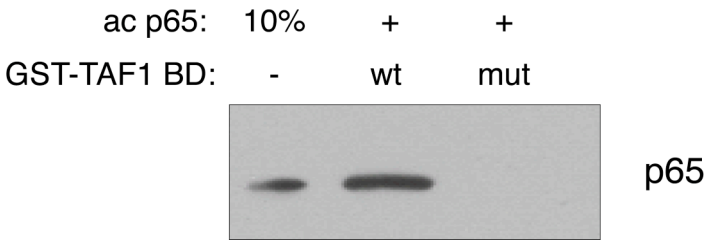


Figure 13. Recombinant p65 wild type was acetylated *in vitro* by p300 and subsequently incubated with GST-TAF1 BD wild type or mutant. Samples were analyzed by western blot against p65.

Because p65 was acetylated by p300 *in vitro* before interaction studies were performed, p300 was present during the pull-down experiments. To determine whether the observed binding between p65 and GST-TAF1 BD is mediated by p300, pull-down experiments were performed in the presence or absence of p300 or acetyl-CoA. Recombinant p65 wild type expressed in insect cells was already slightly acetylated at K310 without the addition of acetyl-CoA, independent on the presence of p300, probably due to acetylation in insect cells (Figure 14, upper panel). After incubation with p300 and acetyl-CoA, p65 acetylation was dramatically increased. Incubation of these different samples with GST-TAF1 BD revealed that the binding between p65 and TAF1 increases in the presence of acetyl-CoA and p300 (Figure 14, lower panel).

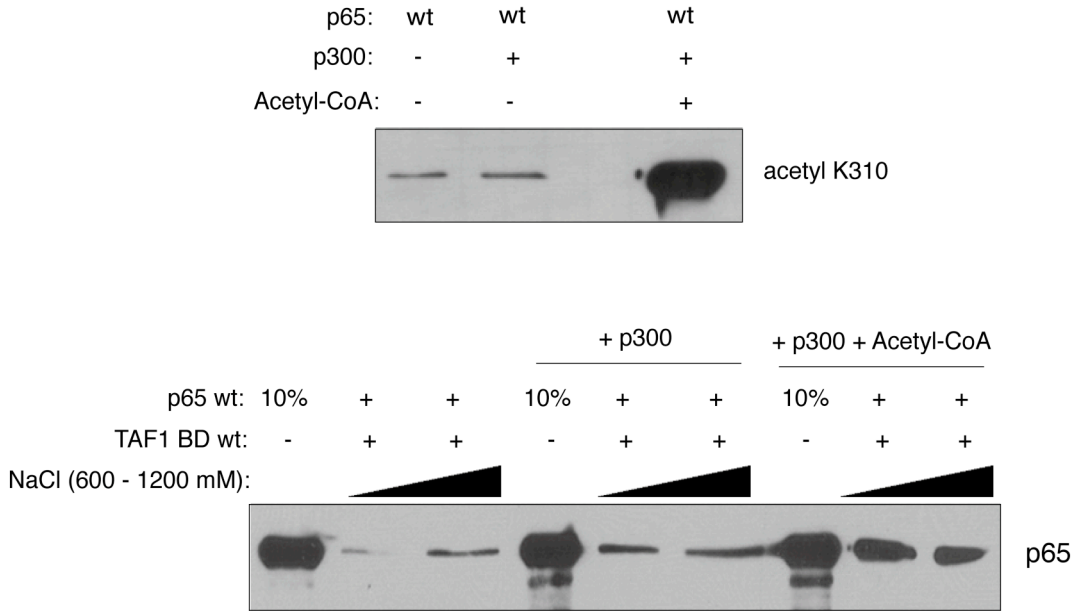


Figure 14. Recombinant purified p65 wild type was incubated alone, with p300 alone or with both p300 and acetyl-CoA (upper panel). These different samples were subsequently incubated with GST-TAF1 BD wild type, washed with binding buffer containing 600 or 1200 mM NaCl and analyzed by western blot (lower panel).

7.3.3 Brd4

Brd4 is a mammalian protein that contains two tandem bromodomains and binds to acetylated histones H3 and H4 [270]. We investigated whether p65 and Brd4 bind in an acetylation-dependent manner *in vivo* by performing co-immunoprecipitation of myc-tagged p65 from the complemented cell lines pTV, wt and KTR treated with HDACi and TNFα. Western blot analysis with an α-Brd4 antibody revealed that Brd4 was unspecifically co-immunoprecipitated even in pTV cells, which do not contain myc-tagged p65 (Figure 15).

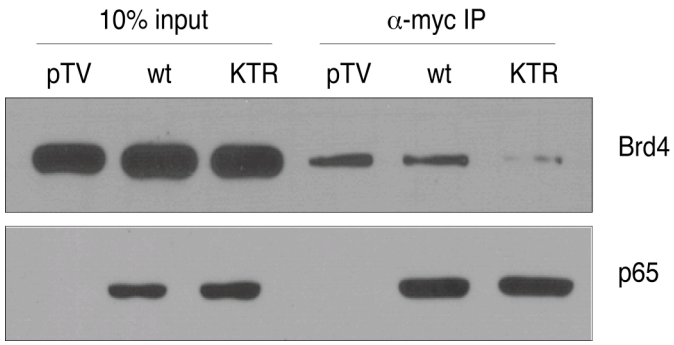


Figure 15. *In vivo interaction between Brd4 and p65.* Complemented MEFs pTV, wt and KTR were treated for 1.5 hour with HDACi TSA and Nam before additional 20 minutes of TNFα stimulation. Nuclear extracts were prepared and myc-tagged p65 was immunoprecipitated form there. Co-immunoprecipitated Brd4 was analyzed by western blot.

The experiment was repeated using α-p65 or α-Brd4 antibodies for the immunoprecipitation, but unfortunately similar results were generated (data not shown). Thus, under the tested conditions, we did not detect a specific interaction between p65 and Brd4 *in vivo*.

7.4 Gene expression in *Sirt2*$^{-/-}$ MEFs

We identified SIRT2 as a novel p65 deacetylase (see section 6.2). Since p65 acetylation influences NF-κB-dependent transcription [245], we decided to investigate whether the expression of NF-κB target genes is affected in *Sirt2*$^{-/-}$ cells *in vivo*. In the submitted manuscript "Deacetylation of p65 by cytoplasmic SIRT2 regulates NF-κB" we describe the misregulation of *Mpa2l*, *RELA* and *Mmp13* in *Sirt2*$^{-/-}$ MEFS compared to *Sirt2*$^{+/+}$ MEFs. Here, we analyzed the expression of three additional NF-κB target genes: *IP10, Cox2* and *Nos2* (Figure 17). In agreement with our reported results, the TNFα-mediated induction of these three genes was higher in *Sirt2*$^{-/-}$ cells as well.

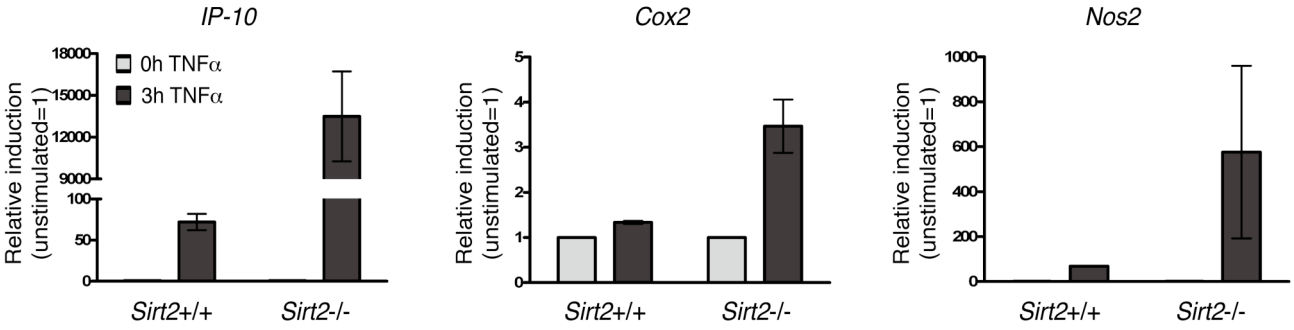


Figure 17. *Misregulated gene expression in Sirt2*$^{-/-}$ *cells*. mRNA levels from *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs were measured by real-time RT-PCR in response to TNFα. The mean value of at least two biological replicates is shown in the graphs ± SD.

7.5 PARP1 protein levels in SIRT1 and SIRT2 knock out MEFs

During western blot analysis of lysates from *Sirt1*$^{+/+}$, *Sirt1*$^{-/-}$, *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs, we realized that the levels of PARP1 were lower in SIRT1 and SIRT2 knock out cells (Figure

18). The major difference was observed between *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ cells (Figure 18, left panel), suggesting that PARP1 protein expression might be affected by SIRT1 and SIRT2.

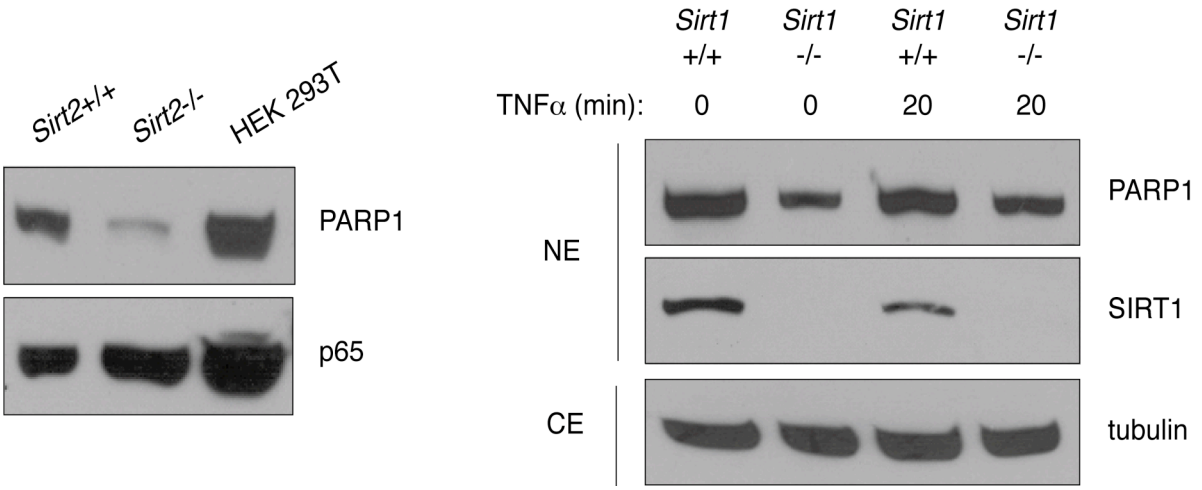


Figure 18. (Left panel) Total cell extracts were prepared from unstimulated *Sirt2*$^{+/+}$, *Sirt2*$^{-/-}$ and HEK 293T cells and analyzed by western blot using the indicated antibodies. **(Right panel)** Nuclear (NE) and cytoplasmic (CE) extracts from *Sirt1*$^{+/+}$and *Sirt1*$^{-/-}$ MEFs left untreated or treated for 20 minutes with TNFα were resolved on SDS-PAGE and analyzed by western blot. Tubulin served as loading control.

7.6 Materials and methods

7.6.1 Endogenous p65 acetylation at K310 after TNFα and IL-1β

Wild type complemented MEFs were starved overnight in medium containing 0% FCS. Cells were left untreated or treated with HDACi (HDACi: 2 mM TSA, 5mM NAM) for 30 minutes before stimulation with 30 ng/ml TNFα or 10 ng/ml IL-1β for 45 additional minutes. Total cell extracts were prepared and used for immunoprecipitation of p65. Samples were resolved on SDS-PAGE and analyzed by western blot using α-acetyl K310 antibody. The membranes were reprobed with α-p65 antibody.

7.6.2 In vitro acetylation assay

One microgram of recombinant human wild type or mutant p65 was incubated with or without 0.5 μg recombinant p300 in HAT buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl,

10% glycerol, 1 mM DTT, 1 mM PMSF, 1 mg/ml pepstatin, 1 mg/ml bestatin, 1 mg/ml leupeptin, 1 mM sodium butyrate) supplemented with or without 5 mM cold acetyl CoA for 1 hour at 30°C. Reactions were stopped by adding 10x Laemmli-buffer and proteins resolved on SDS–PAGE with subsequent western blot analysis using α-acetyl K314/315 or α-acetyl K310 antibodies. The membranes were reprobed for p65.

7.6.3 p65 acetylation in cells

Myc-tagged p65 wild type or K314/315R mutant were co-expressed with p300 in HEK 293T cells. After 24 hours of transfection, cells were treated with HDACi (2 mM TSA, 5mM NAM) for 40 minutes and subsequently stimulated with 10 ng/ml human TNFα (SIGMA) for 30 additional minutes. Whole cell extracts were prepared (50 mM Hepes pH 7.9, 420 mM NaCl, 0.5% NP-40, 1 mM PMSF, 0.5 mM DTT, 1 mg/ml pepstatin, 1 mg/ml bestatin, 1 mg/ml leupeptin, 5 mM TSA, 0.5 M Nam). 30 μg of whole cell extract was resolved on SDS-PAGE and analyzed by standard western blot using α-acetyl K314/315 antibody, followed by reprobe with α-myc antibody.

7.6.4 Immunostaining

Complemented cells were plated at the density of 80 000 cells per chamber. After 6 hours, cells were either left untreated, treated with HDACi (0.5 mM TSA and 0.5 M Nam) alone for 1 hour, or treated with HDACi for 40 minutes and 20 additional minutes with 30 ng/ml mouse TNFα. Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton-X-100/PBS. After blocking for 1 hour in 2% BSA/0.1% Triton-X-100/PBS, samples were incubated with α-p65 antibody (1:250 dilution, Santa Cruz) or α-acetyl K314/315 serum (1:250 dilution), followed by α-rabbit Cy3 antibody (1:250 dilution, Jackson Immunology). The samples were washed and Vectashield mounting solution (Vector laboratories) was applied to prevent bleaching. Cells were visualized using an Olympus T50 microscope.

7.6.5 GST pull-downs

GST-tagged fragment of the double bromodomain from TAF1 (GST-TAF1 BD) was expressed in bacteria. GST-TAF1 BD (wild type or mutant) was immobilized on glutathione beads (Amersham Pharmacia) and incubated with *in vitro* acetylated recombinant p65 (wild type, KTR or KQR mutants) in binding buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40, 1mM PMSF and 1 mg/ml pepstatin, 1 mg/ml bestatin, 1 mg/ml leupeptin, 5 mM Nam, 1 mM TSA) for 2 h at 4°C rolling. Glutathione beads were washed with binding buffer containing distinct NaCl concentrations. Proteins were boiled, resolved on SDS–PAGE and subjected to western blot analysis using α-p65 antibody (C-20, Santa Cruz).

GST-tagged full-length p65 was expressed in insect cells, immobilized on glutathione beads (Amersham Pharmacia) and *in vitro* acetylated by p300. After several washes to get rid of p300, GST-p65 was incubated with His-p300 BD in binding buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40, 1mM PMSF and 1 mg/ml pepstatin, 1 mg/ml bestatin, 1 mg/ml leupeptin, 5 mM Nam, 1 mM TSA) for 3 h at 4°C rolling. Glutathione beads were washed with binding buffer; proteins were boiled and resolved on SDS–PAGE. The gel was fixed, dryed and exposed to X-ray films (Contatyp) at –80°C.

7.6.6 In vitro translation

His-tagged p300 bromodomain (His-p300 BD) was *in vitro* translated using the 'TNT coupled reticulocyte lysate systems' (Promega) following the manufactures instructions.

7.6.7 mRNA quantification by real-time RT-PCR

Total RNA from untreated or 3 hours TNFα-treated *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs was reverse transcribed using the High Capacity cDNA Archive Kit (ABI) according to manufacturers protocol. Real-time RT-PCR was performed using mouse-specific TaqMan probes (Gene expression assays, ABI) for *IP-10, Nos2* and *Cox2*. TaqMan probe for *Rps6* was used to normalize for differences in RNA input. Rotor-Gene3000A (Corbett Life Science, now Qiagen) was used to perform the real-time RT-PCR reactions and the REST program was applied for analysis. The graphs show the mean values of at least two biological replicates ± standard error.

DISCUSSION

8 Summary of results

The aim of this thesis was to examine the role of p65 acetylation; more specifically, its influence on NF-κB-dependent gene expression. We showed that p300 acetylates p65 at lysines 310, 314 and 315 *in vitro* and *in vivo* in response to TNFα. Furthermore, using *p65*$^{-/-}$ MEFs genetically complemented with non-acetylatable mutants, we demonstrated that site-specific acetylation of p65 regulates the expression of particular subsets of NF-κB target genes. Gene expression analysis of the different complemented cell lines suggested that acetylation of K310 is required for the full activation of specific genes, while lysines K314/ K315 are needed for the reduced expression of another subset of genes. Importantly, acetylated p65 was recruited to the promoter of some of these genes, thus providing additional evidence that p65 acetylation is implicated in gene-specific transcriptional regulation.

In the second manuscript (submitted), we also describe SIRT2 as a novel p65 deacetylase after TNFα-induced NF-κB activation. Since we detected the interaction of both endogenous proteins in the cytoplasm and since we showed that SIRT2 stays in that cellular compartment during NF-κB activation by TNFα, our data strongly suggest that p65 is deacetylated by SIRT2 in the cytoplasm after termination of the first wave of NF-κB activation.

In the last manuscript (submitted), the role of CARM1 as a transcriptional coactivator of NF-κB was studied. Custom microarray and real-time RT-PCR experiments identified a subset of NF-κB-dependent genes that require CARM1 for their proper expression. Its enzymatic activity was dispensable, suggesting that CARM1 might be required for the recruitment of other important coactivator or for stabilization of complexes, rather that for methylation of histones or non-histone proteins.

9 Acetylation of p65 and its influence on NF-κB-dependent transcription

The investigation of the effect of p65 acetylation on the NF-κB induction started many years ago in our lab. To study the functional relevance of p65 acetylation, *p65*$^{-/-}$ MEFs were genetically complemented with p65 wild type or acetylation deficient mutants, including a single mutant (K310R), a double mutant (K314/315R) and a triple mutant (KTR). To hinder acetylation in these mutants, the acetylation sites were mutated to arginines. Using these cells, it was shown that p65 acetylation does not affect NF-κB DNA binding, nuclear translocation or IκBα degradation. Interestingly, a difference in gene expression upon TNFα treatment was observed in the different mutants compared to wild type cells (see section 9.1).

As already stated in section 4.4, two other groups have identified additional acetylation sites of p65. Cheng *et al*. reported acetylation at lysines 218, 221 and 310; while the Benkirane group reported lysines 122 and 123 to be modified [244, 261]. Both groups described particular effects on p65 activity by acetylation at the different lysine residues. The discrepancy in the identification of acetylated lysines in p65 by the distinct groups may be explained by the use of different experimental approaches (mass spectrometry vs. peptide mapping analysis), stimuli (TNFα vs. PMA and unstimulated) and cell lines (MEFs vs. HEK 293T, HeLa and Jurkat T-cells).

Although we have demonstrated that lysines 310, 314 and 315 are acetylated *in vitro* and *in vivo* when p300 is co-expressed, we could show p65 acetylation at endogenous levels only for K310 after TNFα stimulation, but not for K314 or K315 acetylation. This suggests that either the quality and sensitivity of the different antibodies is not comparable, that K310 is the major acceptor site for acetylation upon TNFα treatment in MEFs and that acetylation at K314/315 cannot be detected by western blot, or that these lysines are methylated (see section 9.2).

9.1 Regulation of NF-κB-dependent transcription by p65 acetylation

Genome-wide microarray studies revealed that specific genes were either up- or downregulated in the triple mutant KTR compared to wild type cells after 45 minutes of stimulation. In addition, when the cells were stimulated for a longer period, 3 hours, the list

of differentially expressed genes changed, suggesting that transcriptional regulation through p65 acetylation is a dynamic process.

When gene expression profiles of pTV (*p65*$^{-/-}$ MEFs complemented with the empty vector) and wild type cells were compared, we found that the transcriptional activation of several differentially expressed genes between the acetylation-deficient mutants and wild type MEFs did not depend on p65. This could be due to the partly redundancy in gene activation observed between the different NF-κB family members [204]. For example, some genes have been shown to recruit all NF-κB proteins with no apparent specificity to their promoters in dendritic cells [212]. In contrast, another study described that some NF-κB target genes have remarkably different requirements for NF-κB proteins [208]. To avoid any misinterpretation of our results, we decided to focus only on genes whose expression requires p65.

Interestingly, p65 acetylation did not always correlate with transcriptional activation; some of the genes appeared to be less induced in wild type cells compared to non-acetylatable mutants. Although the majority of evidence describes p65 as a transcriptional activator, it has been shown that it can also act as a transcriptional repressor [271, 272]. Gene expression analysis by real-time RT-PCR revealed that the expression of specific genes was increased in the double mutant K314/315R relative to wild type cells, while expression of another subset of genes was reduced when lysine 310 was mutated to arginine (in K310R or KTR cell lines) compared to wild type cells. The requirement of an intact acetylation site at K310 suggests that acetylation at this lysine is needed for full activation of a subset of genes. On the contrary, lysines 314 and/or 315 appear to be required for the tight control of expression of specific genes after induction. Together, acetylation of p65 seems to have opposing effects on gene transcription depending on the lysine residues that are modified. The possible mechanism how this can take place will be further discussed in section 9.2.

The majority of genes found to depend on p65 acetylation were not previously described as NF-κB target genes. However, the impaired induction of several of these genes in pTV suggested that these might be novel NF-κB-dependent genes. To confirm this hypothesis, we performed ChIP assays using primers directed to putative κB sites identified by bioinformatic analysis on the promoters of three genes. In response to TNFα, p65 was recruited to the promoters of *Mpa2l* and *Cfb*. Using antibodies against acetyl K310 and acetyl

K314, we demonstrated that chromatin-associated p65 is acetylated at these two lysines, further implying that acetylation of p65 is involved in transcriptional regulation. ChIP assays with anti-acetyl K315 antibody were unsuccessful. The fact that p65 was found to be acetylated at lysine 314 at the promoters of *Cfb* and *Mpa2l* although the expression of these two genes was not impaired in K314/315R cells suggests that p65 might be acetylated at many more promoters than the ones that really require this modification for proper gene expression.

Our gene expression analysis showed that some genes were regulated by p65 acetylation although it was not recruited to their promoters. Because several transcriptional regulators are among the target genes of NF-κB, it is known that this family of transcription factors influences the expression of many more genes than its direct targets [196]. One example is *Mmp13*, whose transcriptional activation has been shown to require the NF-κB-dependent expression of the transcription factor Elk-1 [273]. Therefore, acetylation of p65 might indirectly regulate the expression of some genes. *Mmp10* appears to be such a gene, since we failed to detect an enrichment of p65 at its promoter. Alternatively, it could be that p65 is recruited to Mmp10 promoter with a different kinetics than the one we investigated; or that p65 binds to an enhancer element localized far away from the TSS of *Mmp10* or even at another chromosome.

9.2 Possible mechanisms of transcriptional regulation by p65 acetylation

The transcriptional activity of several transcription factors has been reported to be modulated by acetylation; the best example of this is p53 [274]. How can the acetylation status of p65 influence the expression of specific NF-κB target genes? This regulation most probably takes place at the chromatin level, since we showed that p65 acetylated at K310 and K314 is bound to chromatin.

Acetyl lysines are known to be recognized by bromodomains. Several transcriptional regulators, such as components of chromatin-remodeling complexes, general transcription factors and HATs, have been reported to get recruited to chromatin through binding of their bromodomains to acetylated histones or other acetylated chromatin-bound proteins (discussed in section 3.3) [114]. The investigation of the interaction between acetyl lysines and bromodomains is difficult to perform, since these interactions are thought to be transient

due to the reversible nature of lysine acetylation. Notably, the affinity of several bromodomain binding to acetyl lysines is generally much weaker than that of chromodomain or PHD finger to methyl lysines [275]. Acetylated p53 is an example of a modified protein recognized by bromodomains. Acetylation of p53 at two lysines has been shown to facilitate the recruitment of TAF1, a subunit of TFIID, to the p21 promoter through its bromodomains [269]. A similar mechanism could be predicted for p65 acetylation. According to our results, we would expect a transcriptional coactivator to bind to acetylated K310. However, interaction studies between acetylated p65 and TAF1 bromodomains in section 7.3.2 revealed that although p65 binding to TAF1 bromodomains increased upon acetylation and was dependent on the integrity of TAF1 double bromodomain, this interaction was not mediated by p65 acetylation at lysines 218, 221, 310, 314 and 315. It could be that TAF1 double bromodomain recognizes p65 acetylated at yet unidentified lysines. Further experiments need to be performed to clarify this issue.

In addition to TAF1, we investigated the interaction between acetylated p65 and the bromodomain of p300. We unexpectedly observed a preferred binding of non-acetylated p65 over acetylated p65 to this bromodomain (section 7.3.1). It could be that the bromodomain of p300 preferentially recognizes non-acetylated lysines of p65, in analogy to the preferential binding of PHD finger of BHC80 to unmethylated H3K4 [98]. Nevertheless, co-immunoprecipitation studies revealed that full-length p300 interacted with p65 wild type at the same extent as with p65 acetylation-deficient mutants [245], suggesting that the acetylation status of p65 is not important for the binding to full-length p300 *in vivo*. Moreover, the bromodomain of p300 is located in the middle of the protein, but p65 has been described to bind to the N-terminal part of p300, further suggesting that acetylation of p65 does not influence binding to p300 *in vivo*.

Recently, Huang *et al.* reported that bromodomains of Brd4 bind to acetylated K310, which enhances transcriptional activation of NF-κB and the expression of a subset of NF-κB inflammatory genes in an acetylated K310-dependent manner [242]. Our results shown in section 7.3.3 failed to confirm this interaction *in vivo*, since we always observed an unspecific band in our negative control pTV. It should be noted that this control is missing in the mentioned study by Huang *et al.*

Another possibility to explain how p65 acetylation modulates gene expression is that site-specific acetylation of p65 interferes with stabilization of the general transcription

machinery, coactivators or corepressors. Furthermore, crosstalk between p65 acetylation and another posttranslational modification in p65 could influence regulation of gene expression. For example, phosphorylation of serine 276 and 536 in p65 have been shown to be needed for binding to p300 [260], and thus required for subsequent acetylation at K310 [262]. Two additional phosphorylation sites in p65 (S311 and S316) are located directly adjacent to the identified acetylated lysine residues, which could influence acetylation at K310, K314 or K315. Moreover, during the writing of this thesis, K314/315 were described to be methylated by Set9 to negatively regulate NF-κB-dependent transcription [276]. Thus, in addition to acetylation, lysines 314 and 315 might be subjected to methylation. This raises the possibility that a crosstalk between these two posttranslational modifications at K314/315 exists, which could influence regulation of gene expression. Our ChIP experiments clearly show that chromatin-bound p65 is acetylated at K310 and K314 in a promoter-specific manner. Based on this observation, we reason that acetylation at K314/315 could prevent methylation-mediated repression of target genes and thus positively influence transcription, together with acetylated lysine 310. A direct evidence for methylated p65 at K314/315 bound to chromatin is still missing, making the interpretation of the results difficult. Importantly, K314/315R mutant MEFs from the mentioned study show increased gene expression compared to wild type cells, which correlates with our results. Whether the same genes are regulated by both posttranslational modifications should be further studied. Alternatively, both posttranslational modifications might regulate distinct set of NF-κB-dependent genes and thus not influence each other.

10 Deacetylation of p65 by SIRT2

Lysine acetylation is a very dynamic process, the steady-state equilibrium of which is regulated by the opposing activities of HATs and HDACs [277]. p65 is not an exception of this: its acetylation status changes rapidly over time. Western blot analysis of *p65*$^{-/-}$ MEFs complemented with p65 wild type and stimulated with TNFα for different time periods uncovered that p65 is acetylated rapidly in the nucleus at lysine 310 (after 20 minutes of stimulation). Acetylation levels were dramatically decreased only 25 minutes after this

observation (after 45 minutes of TNFα treatment). This observation prompted us to investigate which enzyme is responsible for p65 deacetylation.

To explore this, we performed deacetylation assays with overexpressed proteins in HEK 293T cells and with recombinant proteins *in vitro* using class I HDAC members (HDAC1, 2 and 3) and SIRT1, 2, 6 and 7. We chose class I HDAC members because they are known to localize exclusively to the nucleus [144]. SIRT1, 2, 6 and 7 were selected because these class III HDACs have been reported to localize to nucleus and cytoplasm, but not to mitochondria, as SIRT3, 4 and 5 [163]. *In vitro* deacetylation assays with class I HDACs were not performed, since they are known to catalyze deacetylation reactions only in complex with other proteins. In the tested conditions, we observed deacetylation of p65 at all three lysines only by SIRT1 and SIRT2. In contrast to our results, HDAC3 has been previously reported to deacetylate p65 when overexpressed in COS-7 cells [227]. The difference in cell lines used for this and our experiment could explain the discrepancy in both results. Recently, a study showed that SIRT6 does not deacetylate p65 *in vitro* or *in vivo*, in agreement with our results, but deacetylates histone H3K9 to attenuate NF-κB signaling [169]. Our observation that both SIRT1 and SIRT2 were able to deacetylate p65 *in vitro*, but that only deficiency of SIRT2 (but not SIRT1) was sufficient to induce hyperacetylation of p65 at K310 upon TNFα stimulation *in vivo,* suggests that SIRT2 is the main deacetylase of p65 in MEFs upon TNFα stimulation. Consistent with our results, SIRT1 has been shown to deacetylate p65 at K310 *in vitro* and when overexpressed in HEK 293T cells [159]. Thus, it is possible that endogenous SIRT1 plays a major role in deacetylating p65 in other cell types or after NF-κB induction with another stimuli than TNFα. The *in vivo* study of p65 deacetylation by SIRT2 was only possible for acetylated K310, since we were not able to detect endogenous p65 acetylation at K314 or K315 with specific antibodies in MEFs. Thus, we can currently not exclude that another enzyme deacetylates p65 at K314 or K315 *in vivo*.

Several studies have reported that SIRT2 localizes to the cytoplasm, where it deacetylates α-tubulin [161]. Despite its initial description as a cytoplasmic protein, it was later shown that SIRT2 deacetylates nuclear proteins as well, including p53, p300, H3 and H4 [162, 166, 278]. Immunostaining experiments revealed that SIRT2 constantly shuttles between nucleus and cytoplasm during interphase, due to a putative NES found in the N-terminal part of SIRT2, which causes the active export of this sirtuin from the nucleus in a Crm1-dependent manner [164]. Another study described that SIRT2 translocates to the

nucleus at prophase during G2/M transition to deacetylate histone H4 at lysine 16 (H4K16), which results in compaction of the chromatin [162]. Knowing that SIRT2 is predominantly a cytoplasmic protein that can potentially localize to the nucleus, we decided to investigate in which cellular compartment is p65 deacetylated by SIRT2. Due to the nuclear localization of acetylated p65, we investigated whether SIRT2 would translocate to the nucleus upon TNFα, the stimulus that induces p65 acetylation. Immunostaining revealed that this is not the case, SIRT2 stays in the cytoplasm while p65 shuttles to the nucleus upon TNFα stimulation to activate transcription. In addition, we observed that these two proteins interact in the cytoplasm, suggesting that SIRT2 deacetylates p65 in that cellular compartment.

TNFα is known to activate the canonical NF-κB pathway, where p65/p50 heterodimers translocate to the nucleus after IκBα proteosomal degradation. p65/p50 then bind to κB sites in the promoter and enhancer region of regulated genes and recruit coactivators to activate transcription, such as p300. We have shown that p65 is rapidly acetylated by p300 in the nucleus, most probably after the first interaction between these two proteins. Immunostaining and EMSA experiments have shown that the majority of NF-κB shuttles back to the cytoplasm between 30 to 60 minutes after TNFα stimulation in MEFs [245, 279]. This time correlates with the observed decrease in p65 acetylation at K310 with western blot analysis, supporting the idea that p65 is deacetylated in the cytoplasm. Thus, an attractive possibility is that SIRT2 deacetylates p65 when NF-κB shuttles back to the cytoplasm, to reset the NF-κB response. Any p65 that would afterwards translocate to the nucleus again during persistent TNFα stimulation would have to be freshly acetylated by p300 to modulate gene expression, thereby allowing a tight control of NF-κB-dependent transcription.

If SIRT2 is the major enzyme deacetylating p65 after termination of the first wave of NF-κB activation by TNFα, and knowing that p65 acetylation modulates transcription of target genes, then NF-κB-dependent gene expression should be misregulated in the absence of SIRT2. This is indeed the case in *Sirt2*$^{-/-}$ MEFs, where induction of *Mpa2l* is drastically increased in response to TNFα compared to *Sirt2*$^{+/+}$ cells. Since acetylation of p65 at lysine 310 was shown to be required for full activation of this gene and since p65 is hyperacetylated at that lysine in *Sirt2*$^{-/-}$ MEFs, our data suggests that p65 deacetylation by SIRT2 influences NF-κB-dependent transcription of specific genes *in vivo*. Gene expression analysis of a

number of genes by real-time RT-PCR revealed that the induction of all these genes except the control genes *Rps6* and *Rps12* was slightly higher (around 3 fold) in *Sirt2*$^{-/-}$ cells after TNFα treatment compared to *Sirt2*$^{+/+}$ cells (see section 7.5). Considering that SIRT2 has been shown to deacetylate H4K16 to condense chromatin [162], a plausible explanation is that *Sirt2*$^{-/-}$ MEFs possess a more open chromatin structure than wild type MEFs, which would moderately facilitate the recruitment of transcription regulators to chromatin templates.

11 Perspectives

The exact mechanism behind the regulation of NF-κB-dependent transcription by p65 acetylation should be further investigated at the chromatin level. It would be interesting to elucidate by ChIP whether p65 recruits specific bromodomain-containing coactivators or corepressor through its acetyl lysines to the promoters or enhancers of the regulated genes. Interaction studies between acetylated p65 and bromodomain-containing proteins would further help to characterize this interaction. As mentioned above, acetylated K310 could be recognized by the bromodomain of a coactivator needed for the full transcription of *Mpa2l* and *Cfb*, for example. Conversely, acetylation at K310 could hinder the binding of a corepressor to these promoters. Although EMSA studies showed that p65 binding to DNA is not impaired in the acetylation-deficient mutants, the stability of p65/DNA complex should be further addressed *in vivo* by ChIP in the *p65*$^{-/-}$ MEFs complemented with p65 wild type or the acetylation-deficient mutants. It is possible that p65 is not properly recruited to the promoters of *Mpa2l* and *Cfb* when it is not acetylated at K310. In this regard, p65 K314/315R mutant has recently been suggested to bind stronger to chromatin than p65 wild type [276], which should be confirmed in our system. In addition, it may be important to investigate whether there is a crosstalk between the newly described methylation of K314/315 and acetylation at all three lysines 310, 314 and 315.

To understand how general the regulation of NF-κB-dependent transcription by p65 acetylation is, ChIP-chip could be performed using the specific antibodies against acetyl K310 and acetyl K314. This would allow the identification of new genes regulated by p65 acetylation, although we have evidence that acetylated p65 is recruited to many more

promoters than the ones that strictly require it. Furthermore, genome-wide expression analysis after IL-1β stimulation would reveal if p65 acetylation regulates the expression of different genes when distinct stimuli are used. Both TNFα and IL-1β are known to activate the NF-κB response, though in addition, these different cytokines also activate distinct cellular pathways. Thus, it would be interesting to investigate the possible overlap of genes regulated by p65 acetylation upon TNFα and IL-1β. In addition, it should be addressed whether SIRT2 or another enzyme deacetylates p65 after NF-κB activation through IL-1β stimulation. An additional remaining question is whether p65 is acetylated during the non-canonical NF-κB pathway and whether this regulates NF-κB-dependent transcription as well.

Our gene expression analysis in *Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs suggested that overall gene expression is higher in the absence of SIRT2. To confirm this hypothesis, genome-wide expression analysis should be performed with cells from both genotypes, both under basal conditions and after stimulation. In addition, recruitment of acetylated p65 to promoters of regulated genes should be compared in both cell lines, as well as the levels of acetylated H4K16. The difference in chromatin structure between *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ cells could further be studied with MNase protection assays.

It should be noted that when using PARP1 as a nuclear loading control, we unexpectedly observed less expression of this nuclear protein in *Sirt2*$^{-/-}$ cells compared to wild type cells. PARP1 binding to nucleosomes was both reported to promote the formation of compact and transcriptionally repressed chromatin [280], and to correlate with gene expression when it binds to nucleosomes in the promoter of transcribed genes [281]. It would be interesting to investigate why and how PARP1 protein expression is decreased in *Sirt2*$^{-/-}$ MEFS, and whether this observation is linked to chromatin structure in *Sirt2*$^{-/-}$ cells.

NF-κB sustained activation has been correlated with occurrence of some cancers [177]. Specifically, high expression of p65 has been linked to pancreatic cancer [176]. Currently, whether p65 acetylation is involved and even required for tumorogenesis is not known. Future experiments that analyze the acetylation status of p65 in different tumor biopsies should be able to provide insights into this process.

REFERENCES

1. Orphanides G, Reinberg D: **A unified theory of gene expression**. *Cell* 2002, **108**(4):439-451.
2. Roeder RG, Schwartz LB, Sklar VE: **Function, structure, and regulation of eukaryotic nuclear RNA polymerases**. *Symp Soc Dev Biol* 1976(34):29-52.
3. Kornberg RD: **The molecular basis of eucaryotic transcription**. *Cell Death Differ* 2007, **14**(12):1989-1997.
4. Thomas MC, Chiang CM: **The general transcription machinery and general cofactors**. *Crit Rev Biochem Mol Biol* 2006, **41**(3):105-178.
5. Sims RJ, 3rd, Belotserkovskaya R, Reinberg D: **Elongation by RNA polymerase II: the short and long of it**. *Genes Dev* 2004, **18**(20):2437-2468.
6. Young RA: **RNA polymerase II**. *Annu Rev Biochem* 1991, **60**:689-715.
7. Phatnani HP, Greenleaf AL: **Phosphorylation and functions of the RNA polymerase II CTD**. *Genes Dev* 2006, **20**(21):2922-2936.
8. Lolli G: **Binding to DNA of the RNA-polymerase II C-terminal domain allows discrimination between Cdk7 and Cdk9 phosphorylation**. *Nucleic Acids Res* 2009.
9. Egloff S, O'Reilly D, Chapman RD, Taylor A, Tanzhaus K, Pitts L, Eick D, Murphy S: **Serine-7 of the RNA polymerase II CTD is specifically required for snRNA gene expression**. *Science* 2007, **318**(5857):1777-1779.
10. Matsui T, Segall J, Weil PA, Roeder RG: **Multiple factors required for accurate initiation of transcription by purified RNA polymerase II**. *J Biol Chem* 1980, **255**(24):11992-11996.
11. Conaway RC, Conaway JW: **General transcription factors for RNA polymerase II**. *Prog Nucleic Acid Res Mol Biol* 1997, **56**:327-346.
12. Moqtaderi Z, Bai Y, Poon D, Weil PA, Struhl K: **TBP-associated factors are not generally required for transcriptional activation in yeast**. *Nature* 1996, **383**(6596):188-191.
13. Hahn S: **Structure and mechanism of the RNA polymerase II transcription machinery**. *Nat Struct Mol Biol* 2004, **11**(5):394-403.
14. Ptashne M, Gann A: **Transcriptional activation by recruitment**. *Nature* 1997, **386**(6625):569-577.
15. Roeder RG: **Role of general and gene-specific cofactors in the regulation of eukaryotic transcription**. *Cold Spring Harb Symp Quant Biol* 1998, **63**:201-218.
16. Chen L, Widom J: **Mechanism of transcriptional silencing in yeast**. *Cell* 2005, **120**(1):37-48.
17. McKenna NJ, O'Malley BW: **Combinatorial control of gene expression by nuclear receptors and coregulators**. *Cell* 2002, **108**(4):465-474.
18. Malik S, Roeder RG: **Dynamic regulation of pol II transcription by the mammalian Mediator complex**. *Trends Biochem Sci* 2005, **30**(5):256-263.
19. Kim YJ, Bjorklund S, Li Y, Sayre MH, Kornberg RD: **A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II**. *Cell* 1994, **77**(4):599-608.

20. Tsutsui T, Umemura H, Tanaka A, Mizuki F, Hirose Y, Ohkuma Y: **Human mediator kinase subunit CDK11 plays a negative role in viral activator VP16-dependent transcriptional regulation**. *Genes Cells* 2008, **13**(8):817-826.
21. Smale ST, Kadonaga JT: **The RNA polymerase II core promoter**. *Annu Rev Biochem* 2003, **72**:449-479.
22. Maston GA, Evans SK, Green MR: **Transcriptional regulatory elements in the human genome**. *Annu Rev Genomics Hum Genet* 2006, **7**:29-59.
23. Blackwood EM, Kadonaga JT: **Going the distance: a current view of enhancer action**. *Science* 1998, **281**(5373):60-63.
24. Muller MM, Gerster T, Schaffner W: **Enhancer sequences and the regulation of gene transcription**. *Eur J Biochem* 1988, **176**(3):485-495.
25. West AG, Gaszner M, Felsenfeld G: **Insulators: many functions, many mechanisms**. *Genes Dev* 2002, **16**(3):271-288.
26. Bushey AM, Dorman ER, Corces VG: **Chromatin insulators: regulatory mechanisms and epigenetic inheritance**. *Mol Cell* 2008, **32**(1):1-9.
27. Szutorisz H, Dillon N, Tora L: **The role of enhancers as centres for general transcription factor recruitment**. *Trends Biochem Sci* 2005, **30**(11):593-599.
28. Roeder RG: **The role of general initiation factors in transcription by RNA polymerase II**. *Trends Biochem Sci* 1996, **21**(9):327-335.
29. Muller F, Tora L: **The multicoloured world of promoter recognition complexes**. *EMBO J* 2004, **23**(1):2-8.
30. Basehoar AD, Zanton SJ, Pugh BF: **Identification and distinct regulation of yeast TATA box-containing genes**. *Cell* 2004, **116**(5):699-709.
31. Muller F, Demeny MA, Tora L: **New problems in RNA polymerase II transcription initiation: matching the diversity of core promoters with a variety of promoter recognition factors**. *J Biol Chem* 2007, **282**(20):14685-14689.
32. FitzGerald PC, Shlyakhtenko A, Mir AA, Vinson C: **Clustering of DNA sequences in human promoters**. *Genome Res* 2004, **14**(8):1562-1574.
33. Suzuki Y, Tsunoda T, Sese J, Taira H, Mizushima-Sugano J, Hata H, Ota T, Isogai T, Tanaka T, Nakamura Y *et al*: **Identification and characterization of the potential promoter regions of 1031 kinds of human genes**. *Genome Res* 2001, **11**(5):677-684.
34. Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, Ponjavic J, Semple CA, Taylor MS, Engstrom PG, Frith MC *et al*: **Genome-wide analysis of mammalian promoter architecture and evolution**. *Nat Genet* 2006, **38**(6):626-635.
35. Kimura K, Wakamatsu A, Suzuki Y, Ota T, Nishikawa T, Yamashita R, Yamamoto J, Sekine M, Tsuritani K, Wakaguri H *et al*: **Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes**. *Genome Res* 2006, **16**(1):55-65.
36. Kim TH, Barrera LO, Zheng M, Qu C, Singer MA, Richmond TA, Wu Y, Green RD, Ren B: **A high-resolution map of active promoters in the human genome**. *Nature* 2005, **436**(7052):876-880.
37. Koch F, Jourquin F, Ferrier P, Andrau JC: **Genome-wide RNA polymerase II: not genes only!** *Trends Biochem Sci* 2008, **33**(6):265-273.
38. de Laat W, Grosveld F: **Inter-chromosomal gene regulation in the mammalian cell nucleus**. *Curr Opin Genet Dev* 2007, **17**(5):456-464.

39. Osborne CS, Eskiw CH: **Where shall we meet? A role for genome organisation and nuclear sub-compartments in mediating interchromosomal interactions**. *J Cell Biochem* 2008, **104**(5):1553-1561.
40. Gilmour DS, Lis JT: **RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells**. *Mol Cell Biol* 1986, **6**(11):3984-3989.
41. Rougvie AE, Lis JT: **The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged**. *Cell* 1988, **54**(6):795-804.
42. Core LJ, Lis JT: **Transcription regulation through promoter-proximal pausing of RNA polymerase II**. *Science* 2008, **319**(5871):1791-1792.
43. Core LJ, Waterfall JJ, Lis JT: **Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters**. *Science* 2008, **322**(5909):1845-1848.
44. He Y, Vogelstein B, Velculescu VE, Papadopoulos N, Kinzler KW: **The antisense transcriptomes of human cells**. *Science* 2008, **322**(5909):1855-1857.
45. Preker P, Nielsen J, Kammler S, Lykke-Andersen S, Christensen MS, Mapendano CK, Schierup MH, Jensen TH: **RNA exosome depletion reveals transcription upstream of active human promoters**. *Science* 2008, **322**(5909):1851-1854.
46. Seila AC, Calabrese JM, Levine SS, Yeo GW, Rahl PB, Flynn RA, Young RA, Sharp PA: **Divergent transcription from active promoters**. *Science* 2008, **322**(5909):1849-1851.
47. Neil H, Malabat C, d'Aubenton-Carafa Y, Xu Z, Steinmetz LM, Jacquier A: **Widespread bidirectional promoters are the major source of cryptic transcripts in yeast**. *Nature* 2009.
48. Xu Z, Wei W, Gagneur J, Perocchi F, Clauder-Munster S, Camblong J, Guffanti E, Stutz F, Huber W, Steinmetz LM: **Bidirectional promoters generate pervasive transcription in yeast**. *Nature* 2009.
49. Birney E, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingeras TR, Margulies EH, Weng Z, Snyder M, Dermitzakis ET, Thurman RE *et al*: **Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project**. *Nature* 2007, **447**(7146):799-816.
50. Ponting CP, Oliver PL, Reik W: **Evolution and functions of long noncoding RNAs**. *Cell* 2009, **136**(4):629-641.
51. Moazed D: **Small RNAs in transcriptional gene silencing and genome defence**. *Nature* 2009, **457**(7228):413-420.
52. Malone CD, Hannon GJ: **Small RNAs as guardians of the genome**. *Cell* 2009, **136**(4):656-668.
53. Carthew RW, Sontheimer EJ: **Origins and Mechanisms of miRNAs and siRNAs**. *Cell* 2009, **136**(4):642-655.
54. Wahl MC, Will CL, Luhrmann R: **The spliceosome: design principles of a dynamic RNP machine**. *Cell* 2009, **136**(4):701-718.
55. Cam HP, Chen ES, Grewal SI: **Transcriptional scaffolds for heterochromatin assembly**. *Cell* 2009, **136**(4):610-614.
56. Cairney CJ, Keith WN: **Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity**. *Biochimie* 2008, **90**(1):13-23.

57. Kornberg RD, Lorch Y: **Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome**. *Cell* 1999, **98**(3):285-294.
58. Kouzarides T: **Chromatin modifications and their function**. *Cell* 2007, **128**(4):693-705.
59. Kumaran RI, Thakar R, Spector DL: **Chromatin dynamics and gene positioning**. *Cell* 2008, **132**(6):929-934.
60. Sexton T, Schober H, Fraser P, Gasser SM: **Gene regulation through nuclear organization**. *Nat Struct Mol Biol* 2007, **14**(11):1049-1055.
61. Kornberg RD: **Structure of chromatin**. *Annu Rev Biochem* 1977, **46**:931-954.
62. Huisinga KL, Brower-Toland B, Elgin SC: **The contradictory definitions of heterochromatin: transcription and silencing**. *Chromosoma* 2006, **115**(2):110-122.
63. Trojer P, Reinberg D: **Facultative heterochromatin: is there a distinctive molecular signature?** *Mol Cell* 2007, **28**(1):1-13.
64. Kornberg RD: **Chromatin structure: a repeating unit of histones and DNA**. *Science* 1974, **184**(139):868-871.
65. Vaquero A, Loyola A, Reinberg D: **The constantly changing face of chromatin**. *Sci Aging Knowledge Environ* 2003, **2003**(14):RE4.
66. Lodish HF: **Molecular cell biology**, 6th edn. New York: W.H. Freeman; 2007.
67. Mavrich TN, Jiang C, Ioshikhes IP, Li X, Venters BJ, Zanton SJ, Tomsho LP, Qi J, Glaser RL, Schuster SC *et al*: **Nucleosome organization in the Drosophila genome**. *Nature* 2008, **453**(7193):358-362.
68. Schones DE, Cui K, Cuddapah S, Roh TY, Barski A, Wang Z, Wei G, Zhao K: **Dynamic regulation of nucleosome positioning in the human genome**. *Cell* 2008, **132**(5):887-898.
69. Kaplan N, Moore IK, Fondufe-Mittendorf Y, Gossett AJ, Tillo D, Field Y, Leproust EM, Hughes TR, Lieb JD, Widom J *et al*: **The DNA-encoded nucleosome organization of a eukaryotic genome**. *Nature* 2008.
70. Perlmann T, Wrange O: **Specific glucocorticoid receptor binding to DNA reconstituted in a nucleosome**. *EMBO J* 1988, **7**(10):3073-3079.
71. Narlikar GJ, Fan HY, Kingston RE: **Cooperation between complexes that regulate chromatin structure and transcription**. *Cell* 2002, **108**(4):475-487.
72. Wu JI, Lessard J, Crabtree GR: **Understanding the words of chromatin regulation**. *Cell* 2009, **136**(2):200-206.
73. Li B, Carey M, Workman JL: **The role of chromatin during transcription**. *Cell* 2007, **128**(4):707-719.
74. Sterner DE, Berger SL: **Acetylation of histones and transcription-related factors**. *Microbiol Mol Biol Rev* 2000, **64**(2):435-459.
75. Zhang Y, Reinberg D: **Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails**. *Genes Dev* 2001, **15**(18):2343-2360.
76. Nowak SJ, Corces VG: **Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation**. *Trends Genet* 2004, **20**(4):214-220.
77. Weake VM, Workman JL: **Histone ubiquitination: triggering gene activity**. *Mol Cell* 2008, **29**(6):653-663.

78. Hassa PO, Haenni SS, Elser M, Hottiger MO: **Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going?** *Microbiol Mol Biol Rev* 2006, **70**(3):789-829.
79. Nathan D, Ingvarsdottir K, Sterner DE, Bylebyl GR, Dokmanovic M, Dorsey JA, Whelan KA, Krsmanovic M, Lane WS, Meluh PB *et al*: **Histone sumoylation is a negative regulator in Saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications**. *Genes Dev* 2006, **20**(8):966-976.
80. Cuthbert GL, Daujat S, Snowden AW, Erdjument-Bromage H, Hagiwara T, Yamada M, Schneider R, Gregory PD, Tempst P, Bannister AJ *et al*: **Histone deimination antagonizes arginine methylation**. *Cell* 2004, **118**(5):545-553.
81. Shaw PE: **Peptidyl-prolyl cis/trans isomerases and transcription: is there a twist in the tail?** *EMBO Rep* 2007, **8**(1):40-45.
82. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA: **Histone demethylation mediated by the nuclear amine oxidase homolog LSD1**. *Cell* 2004, **119**(7):941-953.
83. Berger SL: **The complex language of chromatin regulation during transcription**. *Nature* 2007, **447**(7143):407-412.
84. Roguev A, Schaft D, Shevchenko A, Pijnappel WW, Wilm M, Aasland R, Stewart AF: **The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4**. *EMBO J* 2001, **20**(24):7137-7148.
85. Nishioka K, Chuikov S, Sarma K, Erdjument-Bromage H, Allis CD, Tempst P, Reinberg D: **Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation**. *Genes Dev* 2002, **16**(4):479-489.
86. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA *et al*: **Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome**. *Nat Genet* 2007, **39**(3):311-318.
87. Briggs SD, Bryk M, Strahl BD, Cheung WL, Davie JK, Dent SY, Winston F, Allis CD: **Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae**. *Genes Dev* 2001, **15**(24):3286-3295.
88. Suganuma T, Workman JL: **Crosstalk among Histone Modifications**. *Cell* 2008, **135**(4):604-607.
89. Lo WS, Trievel RC, Rojas JR, Duggan L, Hsu JY, Allis CD, Marmorstein R, Berger SL: **Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14**. *Mol Cell* 2000, **5**(6):917-926.
90. Lee JS, Shilatifard A: **A site to remember: H3K36 methylation a mark for histone deacetylation**. *Mutat Res* 2007, **618**(1-2):130-134.
91. Lee JS, Shukla A, Schneider J, Swanson SK, Washburn MP, Florens L, Bhaumik SR, Shilatifard A: **Histone crosstalk between H2B monoubiquitination and H3 methylation mediated by COMPASS**. *Cell* 2007, **131**(6):1084-1096.
92. Strahl BD, Allis CD: **The language of covalent histone modifications**. *Nature* 2000, **403**(6765):41-45.
93. Ruthenburg AJ, Li H, Patel DJ, Allis CD: **Multivalent engagement of chromatin modifications by linked binding modules**. *Nat Rev Mol Cell Biol* 2007, **8**(12):983-994.

94. Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ: **How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers**. *Nat Struct Mol Biol* 2007, **14**(11):1025-1040.
95. Jacobson RH, Ladurner AG, King DS, Tjian R: **Structure and function of a human TAFII250 double bromodomain module**. *Science* 2000, **288**(5470):1422-1425.
96. Vermeulen M, Mulder KW, Denissov S, Pijnappel WW, van Schaik FM, Varier RA, Baltissen MP, Stunnenberg HG, Mann M, Timmers HT: **Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4**. *Cell* 2007, **131**(1):58-69.
97. Nielsen PR, Nietlispach D, Mott HR, Callaghan J, Bannister A, Kouzarides T, Murzin AG, Murzina NV, Laue ED: **Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9**. *Nature* 2002, **416**(6876):103-107.
98. Lan F, Collins RE, De Cegli R, Alpatov R, Horton JR, Shi X, Gozani O, Cheng X, Shi Y: **Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression**. *Nature* 2007, **448**(7154):718-722.
99. Sims RJ, 3rd, Reinberg D: **Is there a code embedded in proteins that is based on post-translational modifications?** *Nat Rev Mol Cell Biol* 2008, **9**(10):815-820.
100. Kuo MH, Allis CD: **Roles of histone acetyltransferases and deacetylases in gene regulation**. *Bioessays* 1998, **20**(8):615-626.
101. Mukherjee S, Keitany G, Li Y, Wang Y, Ball HL, Goldsmith EJ, Orth K: **Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation**. *Science* 2006, **312**(5777):1211-1214.
102. Mittal R, Peak-Chew SY, McMahon HT: **Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling**. *Proc Natl Acad Sci U S A* 2006, **103**(49):18574-18579.
103. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB: **Histone deacetylases (HDACs): characterization of the classical HDAC family**. *Biochem J* 2003, **370**(Pt 3):737-749.
104. Phillips DM: **The presence of acetyl groups of histones**. *Biochem J* 1963, **87**:258-263.
105. Allfrey VG, Faulkner R, Mirsky AE: **Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis**. *Proc Natl Acad Sci U S A* 1964, **51**:786-794.
106. Brownell JE, Allis CD: **An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in Tetrahymena macronuclei**. *Proc Natl Acad Sci U S A* 1995, **92**(14):6364-6368.
107. Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, Peterson CL: **Histone H4-K16 acetylation controls chromatin structure and protein interactions**. *Science* 2006, **311**(5762):844-847.
108. Hong L, Schroth GP, Matthews HR, Yau P, Bradbury EM: **Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 "tail" to DNA**. *J Biol Chem* 1993, **268**(1):305-314.
109. Hebbes TR, Thorne AW, Crane-Robinson C: **A direct link between core histone acetylation and transcriptionally active chromatin**. *EMBO J* 1988, **7**(5):1395-1402.

110. Grozinger CM, Schreiber SL: **Deacetylase enzymes: biological functions and the use of small-molecule inhibitors**. *Chem Biol* 2002, **9**(1):3-16.
111. Sterner R, Vidali G, Allfrey VG: **Studies of acetylation and deacetylation in high mobility group proteins. Identification of the sites of acetylation in HMG-1**. *J Biol Chem* 1979, **254**(22):11577-11583.
112. Gu W, Roeder RG: **Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain**. *Cell* 1997, **90**(4):595-606.
113. Glozak MA, Sengupta N, Zhang X, Seto E: **Acetylation and deacetylation of non-histone proteins**. *Gene* 2005, **363**:15-23.
114. Yang XJ: **Lysine acetylation and the bromodomain: a new partnership for signaling**. *Bioessays* 2004, **26**(10):1076-1087.
115. Yang XJ, Seto E: **Lysine acetylation: codified crosstalk with other posttranslational modifications**. *Mol Cell* 2008, **31**(4):449-461.
116. Yang XJ: **The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases**. *Nucleic Acids Res* 2004, **32**(3):959-976.
117. Lee KK, Workman JL: **Histone acetyltransferase complexes: one size doesn't fit all**. *Nat Rev Mol Cell Biol* 2007, **8**(4):284-295.
118. Kleff S, Andrulis ED, Anderson CW, Sternglanz R: **Identification of a gene encoding a yeast histone H4 acetyltransferase**. *J Biol Chem* 1995, **270**(42):24674-24677.
119. Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY, Allis CD: **Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation**. *Cell* 1996, **84**(6):843-851.
120. Mizzen CA, Yang XJ, Kokubo T, Brownell JE, Bannister AJ, Owen-Hughes T, Workman J, Wang L, Berger SL, Kouzarides T *et al*: **The TAF(II)250 subunit of TFIID has histone acetyltransferase activity**. *Cell* 1996, **87**(7):1261-1270.
121. Bannister AJ, Kouzarides T: **The CBP co-activator is a histone acetyltransferase**. *Nature* 1996, **384**(6610):641-643.
122. Roth SY, Denu JM, Allis CD: **Histone acetyltransferases**. *Annu Rev Biochem* 2001, **70**:81-120.
123. Poux AN, Marmorstein R: **Molecular basis for Gcn5/PCAF histone acetyltransferase selectivity for histone and nonhistone substrates**. *Biochemistry* 2003, **42**(49):14366-14374.
124. Yang XJ, Seto E: **HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention**. *Oncogene* 2007, **26**(37):5310-5318.
125. Neuwald AF, Landsman D: **GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein**. *Trends Biochem Sci* 1997, **22**(5):154-155.
126. Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, Candau R, Ohba R, Owen-Hughes T, Allis CD, Winston F *et al*: **Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex**. *Genes Dev* 1997, **11**(13):1640-1650.
127. Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y: **A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A**. *Nature* 1996, **382**(6589):319-324.

128. Yamamoto T, Horikoshi M: **Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60**. *J Biol Chem* 1997, **272**(49):30595-30598.
129. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y: **The transcriptional coactivators p300 and CBP are histone acetyltransferases**. *Cell* 1996, **87**(5):953-959.
130. Thompson PR, Wang D, Wang L, Fulco M, Pediconi N, Zhang D, An W, Ge Q, Roeder RG, Wong J *et al*: **Regulation of the p300 HAT domain via a novel activation loop**. *Nat Struct Mol Biol* 2004, **11**(4):308-315.
131. Goodman RH, Smolik S: **CBP/p300 in cell growth, transformation, and development**. *Genes Dev* 2000, **14**(13):1553-1577.
132. Eckner R, Arany Z, Ewen M, Sellers W, Livingston DM: **The adenovirus E1A-associated 300-kD protein exhibits properties of a transcriptional coactivator and belongs to an evolutionarily conserved family**. *Cold Spring Harb Symp Quant Biol* 1994, **59**:85-95.
133. Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, Newsome D, Bronson RT, Li E, Livingston DM, Eckner R: **Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300**. *Cell* 1998, **93**(3):361-372.
134. Kimura A, Matsubara K, Horikoshi M: **A decade of histone acetylation: marking eukaryotic chromosomes with specific codes**. *J Biochem* 2005, **138**(6):647-662.
135. Kundu TK, Wang Z, Roeder RG: **Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity**. *Mol Cell Biol* 1999, **19**(2):1605-1615.
136. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ *et al*: **Steroid receptor coactivator-1 is a histone acetyltransferase**. *Nature* 1997, **389**(6647):194-198.
137. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM: **Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300**. *Cell* 1997, **90**(3):569-580.
138. Doi M, Hirayama J, Sassone-Corsi P: **Circadian regulator CLOCK is a histone acetyltransferase**. *Cell* 2006, **125**(3):497-508.
139. Driscoll R, Hudson A, Jackson SP: **Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56**. *Science* 2007, **315**(5812):649-652.
140. Taunton J, Hassig CA, Schreiber SL: **A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p**. *Science* 1996, **272**(5260):408-411.
141. Yang XJ, Seto E: **The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men**. *Nat Rev Mol Cell Biol* 2008, **9**(3):206-218.
142. Gao L, Cueto MA, Asselbergs F, Atadja P: **Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family**. *J Biol Chem* 2002, **277**(28):25748-25755.
143. Gregoretti IV, Lee YM, Goodson HV: **Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis**. *J Mol Biol* 2004, **338**(1):17-31.
144. Fischle W, Kiermer V, Dequiedt F, Verdin E: **The emerging role of class II histone deacetylases**. *Biochem Cell Biol* 2001, **79**(3):337-348.

145. Vaquero A, Sternglanz R, Reinberg D: **NAD+-dependent deacetylation of H4 lysine 16 by class III HDACs**. *Oncogene* 2007, **26**(37):5505-5520.
146. Blander G, Guarente L: **The Sir2 family of protein deacetylases**. *Annu Rev Biochem* 2004, **73**:417-435.
147. Michan S, Sinclair D: **Sirtuins in mammals: insights into their biological function**. *Biochem J* 2007, **404**(1):1-13.
148. Saunders LR, Verdin E: **Sirtuins: critical regulators at the crossroads between cancer and aging**. *Oncogene* 2007, **26**(37):5489-5504.
149. Schwer B, Verdin E: **Conserved metabolic regulatory functions of sirtuins**. *Cell Metab* 2008, **7**(2):104-112.
150. Denu JM: **Linking chromatin function with metabolic networks: Sir2 family of NAD(+)-dependent deacetylases**. *Trends Biochem Sci* 2003, **28**(1):41-48.
151. Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, Valenzuela DM, Yancopoulos GD, Karow M, Blander G *et al*: **SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells**. *Cell* 2006, **126**(5):941-954.
152. Liszt G, Ford E, Kurtev M, Guarente L: **Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase**. *J Biol Chem* 2005, **280**(22):21313-21320.
153. Frye RA: **Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity**. *Biochem Biophys Res Commun* 1999, **260**(1):273-279.
154. Shi T, Wang F, Stieren E, Tong Q: **SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes**. *J Biol Chem* 2005, **280**(14):13560-13567.
155. Taylor DM, Maxwell MM, Luthi-Carter R, Kazantsev AG: **Biological and potential therapeutic roles of sirtuin deacetylases**. *Cell Mol Life Sci* 2008, **65**(24):4000-4018.
156. Vaquero A, Scher M, Lee D, Erdjument-Bromage H, Tempst P, Reinberg D: **Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin**. *Mol Cell* 2004, **16**(1):93-105.
157. Bouras T, Fu M, Sauve AA, Wang F, Quong AA, Perkins ND, Hay RT, Gu W, Pestell RG: **SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1**. *J Biol Chem* 2005, **280**(11):10264-10276.
158. Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W: **Negative control of p53 by Sir2alpha promotes cell survival under stress**. *Cell* 2001, **107**(2):137-148.
159. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW: **Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase**. *EMBO J* 2004, **23**(12):2369-2380.
160. Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA: **Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle**. *Mol Cell Biol* 2003, **23**(9):3173-3185.
161. North BJ, Marshall BL, Borra MT, Denu JM, Verdin E: **The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase**. *Mol Cell* 2003, **11**(2):437-444.

162. Vaquero A, Scher MB, Lee DH, Sutton A, Cheng HL, Alt FW, Serrano L, Sternglanz R, Reinberg D: **SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis**. *Genes Dev* 2006, **20**(10):1256-1261.
163. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I: **Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins**. *Mol Biol Cell* 2005, **16**(10):4623-4635.
164. North BJ, Verdin E: **Interphase nucleo-cytoplasmic shuttling and localization of SIRT2 during mitosis**. *PLoS ONE* 2007, **2**(8):e784.
165. Jing E, Gesta S, Kahn CR: **SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation**. *Cell Metab* 2007, **6**(2):105-114.
166. Black JC, Mosley A, Kitada T, Washburn M, Carey M: **The SIRT2 deacetylase regulates autoacetylation of p300**. *Mol Cell* 2008, **32**(3):449-455.
167. Schlicker C, Gertz M, Papatheodorou P, Kachholz B, Becker CF, Steegborn C: **Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5**. *J Mol Biol* 2008, **382**(3):790-801.
168. Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TL, Barrett JC *et al*: **SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin**. *Nature* 2008, **452**(7186):492-496.
169. Kawahara TL, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KC, Boxer LD, Chang HY *et al*: **SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span**. *Cell* 2009, **136**(1):62-74.
170. Ford E, Voit R, Liszt G, Magin C, Grummt I, Guarente L: **Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription**. *Genes Dev* 2006, **20**(9):1075-1080.
171. Vakhrusheva O, Smolka C, Gajawada P, Kostin S, Boettger T, Kubin T, Braun T, Bober E: **Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice**. *Circ Res* 2008, **102**(6):703-710.
172. Jeanmougin F, Wurtz JM, Le Douarin B, Chambon P, Losson R: **The bromodomain revisited**. *Trends Biochem Sci* 1997, **22**(5):151-153.
173. Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, Zhou MM: **Structure and ligand of a histone acetyltransferase bromodomain**. *Nature* 1999, **399**(6735):491-496.
174. Mujtaba S, Zeng L, Zhou MM: **Structure and acetyl-lysine recognition of the bromodomain**. *Oncogene* 2007, **26**(37):5521-5527.
175. Hayden MS, Ghosh S: **Shared principles in NF-kappaB signaling**. *Cell* 2008, **132**(3):344-362.
176. Weichert W, Boehm M, Gekeler V, Bahra M, Langrehr J, Neuhaus P, Denkert C, Imre G, Weller C, Hofmann HP *et al*: **High expression of RelA/p65 is associated with activation of nuclear factor-kappaB-dependent signaling in pancreatic cancer and marks a patient population with poor prognosis**. *Br J Cancer* 2007, **97**(4):523-530.
177. Courtois G, Gilmore TD: **Mutations in the NF-kappaB signaling pathway: implications for human disease**. *Oncogene* 2006, **25**(51):6831-6843.

178. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: **Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B**. *Nature* 1995, **376**(6536):167-170.
179. Sen R, Baltimore D: **Multiple nuclear factors interact with the immunoglobulin enhancer sequences**. *Cell* 1986, **46**(5):705-716.
180. Gilmore TD: **Introduction to NF-kappaB: players, pathways, perspectives**. *Oncogene* 2006, **25**(51):6680-6684.
181. Gilmore TD: **NF-kappa B, KBF1, dorsal, and related matters**. *Cell* 1990, **62**(5):841-843.
182. Hayden MS, Ghosh S: **Signaling to NF-kappaB**. *Genes Dev* 2004, **18**(18):2195-2224.
183. Lin L, DeMartino GN, Greene WC: **Cotranslational biogenesis of NF-kappaB p50 by the 26S proteasome**. *Cell* 1998, **92**(6):819-828.
184. Schmitz ML, Baeuerle PA: **The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B**. *EMBO J* 1991, **10**(12):3805-3817.
185. Bonizzi G, Karin M: **The two NF-kappaB activation pathways and their role in innate and adaptive immunity**. *Trends Immunol* 2004, **25**(6):280-288.
186. Zabel U, Baeuerle PA: **Purified human I kappa B can rapidly dissociate the complex of the NF-kappa B transcription factor with its cognate DNA**. *Cell* 1990, **61**(2):255-265.
187. Wu H, Lozano G: **NF-kappa B activation of p53. A potential mechanism for suppressing cell growth in response to stress**. *J Biol Chem* 1994, **269**(31):20067-20074.
188. Duyao MP, Kessler DJ, Spicer DB, Bartholomew C, Cleveland JL, Siekevitz M, Sonenshein GE: **Transactivation of the c-myc promoter by human T cell leukemia virus type 1 tax is mediated by NF kappa B**. *J Biol Chem* 1992, **267**(23):16288-16291.
189. Bonello S, Zahringer C, BelAiba RS, Djordjevic T, Hess J, Michiels C, Kietzmann T, Gorlach A: **Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site**. *Arterioscler Thromb Vasc Biol* 2007, **27**(4):755-761.
190. Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC *et al*: **Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway**. *Science* 2001, **293**(5534):1495-1499.
191. Solan NJ, Miyoshi H, Carmona EM, Bren GD, Paya CV: **RelB cellular regulation and transcriptional activity are regulated by p100**. *J Biol Chem* 2002, **277**(2):1405-1418.
192. Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, Li ZW, Karin M, Ware CF, Green DR: **The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways**. *Immunity* 2002, **17**(4):525-535.
193. Weih DS, Yilmaz ZB, Weih F: **Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines**. *J Immunol* 2001, **167**(4):1909-1919.
194. Kato T, Jr., Delhase M, Hoffmann A, Karin M: **CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response**. *Mol Cell* 2003, **12**(4):829-839.

195. Tergaonkar V, Bottero V, Ikawa M, Li Q, Verma IM: **IkappaB kinase-independent IkappaBalpha degradation pathway: functional NF-kappaB activity and implications for cancer therapy**. *Mol Cell Biol* 2003, **23**(22):8070-8083.
196. Pahl HL: **Activators and target genes of Rel/NF-kappaB transcription factors**. *Oncogene* 1999, **18**(49):6853-6866.
197. Ghosh S, Hayden MS: **New regulators of NF-kappaB in inflammation**. *Nat Rev Immunol* 2008, **8**(11):837-848.
198. Karin M: **How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex**. *Oncogene* 1999, **18**(49):6867-6874.
199. Liou HC, Sha WC, Scott ML, Baltimore D: **Sequential induction of NF-kappa B/Rel family proteins during B-cell terminal differentiation**. *Mol Cell Biol* 1994, **14**(8):5349-5359.
200. Martone R, Euskirchen G, Bertone P, Hartman S, Royce TE, Luscombe NM, Rinn JL, Nelson FK, Miller P, Gerstein M *et al*: **Distribution of NF-kappaB-binding sites across human chromosome 22**. *Proc Natl Acad Sci U S A* 2003, **100**(21):12247-12252.
201. Lim CA, Yao F, Wong JJ, George J, Xu H, Chiu KP, Sung WK, Lipovich L, Vega VB, Chen J *et al*: **Genome-wide mapping of RELA(p65) binding identifies E2F1 as a transcriptional activator recruited by NF-kappaB upon TLR4 activation**. *Mol Cell* 2007, **27**(4):622-635.
202. Muesing MA, Smith DH, Capon DJ: **Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein**. *Cell* 1987, **48**(4):691-701.
203. Cherrington JM, Mocarski ES: **Human cytomegalovirus ie1 transactivates the alpha promoter-enhancer via an 18-base-pair repeat element**. *J Virol* 1989, **63**(3):1435-1440.
204. Natoli G: **Tuning up inflammation: how DNA sequence and chromatin organization control the induction of inflammatory genes by NF-kappaB**. *FEBS Lett* 2006, **580**(12):2843-2849.
205. Kunsch C, Ruben SM, Rosen CA: **Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation**. *Mol Cell Biol* 1992, **12**(10):4412-4421.
206. Bonizzi G, Bebien M, Otero DC, Johnson-Vroom KE, Cao Y, Vu D, Jegga AG, Aronow BJ, Ghosh G, Rickert RC *et al*: **Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers**. *EMBO J* 2004, **23**(21):4202-4210.
207. Natoli G, Saccani S, Bosisio D, Marazzi I: **Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time**. *Nat Immunol* 2005, **6**(5):439-445.
208. Hoffmann A, Leung TH, Baltimore D: **Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities**. *EMBO J* 2003, **22**(20):5530-5539.
209. Leung TH, Hoffmann A, Baltimore D: **One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers**. *Cell* 2004, **118**(4):453-464.
210. Chen FE, Huang DB, Chen YQ, Ghosh G: **Crystal structure of p50/p65 heterodimer of transcription factor NF-kappaB bound to DNA**. *Nature* 1998, **391**(6665):410-413.

211. Bosisio D, Marazzi I, Agresti A, Shimizu N, Bianchi ME, Natoli G: **A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-kappaB-dependent gene activity**. *EMBO J* 2006, **25**(4):798-810.
212. Saccani S, Pantano S, Natoli G: **Modulation of NF-kappaB activity by exchange of dimers**. *Mol Cell* 2003, **11**(6):1563-1574.
213. Schreiber J, Jenner RG, Murray HL, Gerber GK, Gifford DK, Young RA: **Coordinated binding of NF-kappaB family members in the response of human cells to lipopolysaccharide**. *Proc Natl Acad Sci U S A* 2006, **103**(15):5899-5904.
214. Jones PL, Ping D, Boss JM: **Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB**. *Mol Cell Biol* 1997, **17**(12):6970-6981.
215. Thanos D, Maniatis T: **Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome**. *Cell* 1995, **83**(7):1091-1100.
216. Apostolou E, Thanos D: **Virus Infection Induces NF-kappaB-dependent interchromosomal associations mediating monoallelic IFN-beta gene expression**. *Cell* 2008, **134**(1):85-96.
217. George AA, Sharma M, Singh BN, Sahoo NC, Rao KV: **Transcription regulation from a TATA and INR-less promoter: spatial segregation of promoter function**. *EMBO J* 2006, **25**(4):811-821.
218. Angelov D, Lenouvel F, Hans F, Muller CW, Bouvet P, Bednar J, Moudrianakis EN, Cadet J, Dimitrov S: **The histone octamer is invisible when NF-kappaB binds to the nucleosome**. *J Biol Chem* 2004, **279**(41):42374-42382.
219. Saccani S, Pantano S, Natoli G: **Two waves of nuclear factor kappaB recruitment to target promoters**. *J Exp Med* 2001, **193**(12):1351-1359.
220. Ramirez-Carrozzi VR, Nazarian AA, Li CC, Gore SL, Sridharan R, Imbalzano AN, Smale ST: **Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response**. *Genes Dev* 2006, **20**(3):282-296.
221. Boekhoudt GH, Guo Z, Beresford GW, Boss JM: **Communication between NF-kappa B and Sp1 controls histone acetylation within the proximal promoter of the monocyte chemoattractant protein 1 gene**. *J Immunol* 2003, **170**(8):4139-4147.
222. Genin P, Algarte M, Roof P, Lin R, Hiscott J: **Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors**. *J Immunol* 2000, **164**(10):5352-5361.
223. Zhong H, May MJ, Jimi E, Ghosh S: **The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1**. *Mol Cell* 2002, **9**(3):625-636.
224. Hoberg JE, Yeung F, Mayo MW: **SMRT derepression by the IkappaB kinase alpha: a prerequisite to NF-kappaB transcription and survival**. *Mol Cell* 2004, **16**(2):245-255.
225. Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG: **Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein**. *Cell* 2002, **110**(1):55-67.
226. Lee SK, Kim JH, Lee YC, Cheong J, Lee JW: **Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule**

of activating protein-1, nuclear factor-kappaB, and serum response factor. *J Biol Chem* 2000, **275**(17):12470-12474.
227. Chen L, Fischle W, Verdin E, Greene WC: **Duration of nuclear NF-kappaB action regulated by reversible acetylation**. *Science* 2001, **293**(5535):1653-1657.
228. Ashburner BP, Westerheide SD, Baldwin AS, Jr.: **The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression**. *Mol Cell Biol* 2001, **21**(20):7065-7077.
229. Owen HR, Elser M, Cheung E, Gersbach M, Kraus WL, Hottiger MO: **MYBBP1a is a novel repressor of NF-kappaB**. *J Mol Biol* 2007, **366**(3):725-736.
230. Ramsey CS, Yeung F, Stoddard PB, Li D, Creutz CE, Mayo MW: **Copine-I represses NF-kappaB transcription by endoproteolysis of p65**. *Oncogene* 2008, **27**(25):3516-3526.
231. Gerritsen ME, Williams AJ, Neish AS, Moore S, Shi Y, Collins T: **CREB-binding protein/p300 are transcriptional coactivators of p65**. *Proc Natl Acad Sci U S A* 1997, **94**(7):2927-2932.
232. Perkins ND, Felzien LK, Betts JC, Leung K, Beach DH, Nabel GJ: **Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator**. *Science* 1997, **275**(5299):523-527.
233. Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T: **Transcriptional activation by NF-kappaB requires multiple coactivators**. *Mol Cell Biol* 1999, **19**(9):6367-6378.
234. Na SY, Lee SK, Han SJ, Choi HS, Im SY, Lee JW: **Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor kappaB-mediated transactivations**. *J Biol Chem* 1998, **273**(18):10831-10834.
235. Schmitz ML, Stelzer G, Altmann H, Meisterernst M, Baeuerle PA: **Interaction of the COOH-terminal transactivation domain of p65 NF-kappa B with TATA-binding protein, transcription factor IIB, and coactivators**. *J Biol Chem* 1995, **270**(13):7219-7226.
236. Yamit-Hezi A, Dikstein R: **TAFII105 mediates activation of anti-apoptotic genes by NF-kappaB**. *EMBO J* 1998, **17**(17):5161-5169.
237. Guermah M, Malik S, Roeder RG: **Involvement of TFIID and USA components in transcriptional activation of the human immunodeficiency virus promoter by NF-kappaB and Sp1**. *Mol Cell Biol* 1998, **18**(6):3234-3244.
238. Covic M, Hassa PO, Saccani S, Buerki C, Meier NI, Lombardi C, Imhof R, Bedford MT, Natoli G, Hottiger MO: **Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression**. *EMBO J* 2005, **24**(1):85-96.
239. Hassa PO, Hottiger MO: **A role of poly (ADP-ribose) polymerase in NF-kappaB transcriptional activation**. *Biol Chem* 1999, **380**(7-8):953-959.
240. Tetsuka T, Uranishi H, Sanda T, Asamitsu K, Yang JP, Wong-Staal F, Okamoto T: **RNA helicase A interacts with nuclear factor kappaB p65 and functions as a transcriptional coactivator**. *Eur J Biochem* 2004, **271**(18):3741-3751.
241. Barboric M, Nissen RM, Kanazawa S, Jabrane-Ferrat N, Peterlin BM: **NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II**. *Mol Cell* 2001, **8**(2):327-337.

242. Huang B, Yang X, Zhou MM, Ozato K, Chen LF: **Brd4 Coactivates Transcriptional Activation of NF-{kappa}B Via Specific Binding to Acetylated RelA**. *Mol Cell Biol* 2008.
243. Chan HM, La Thangue NB: **p300/CBP proteins: HATs for transcriptional bridges and scaffolds**. *J Cell Sci* 2001, **114**(Pt 13):2363-2373.
244. Chen LF, Mu Y, Greene WC: **Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB**. *EMBO J* 2002, **21**(23):6539-6548.
245. Buerki C, Rothgiesser KM, Valovka T, Owen HR, Rehrauer H, Fey M, Lane WS, Hottiger MO: **Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65**. *Nucleic Acids Res* 2008, **36**(5):1665-1680.
246. Hassa PO, Haenni SS, Buerki C, Meier NI, Lane WS, Owen H, Gersbach M, Imhof R, Hottiger MO: **Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription**. *J Biol Chem* 2005, **280**(49):40450-40464.
247. Bedford MT, Clarke SG: **Protein arginine methylation in mammals: who, what, and why**. *Mol Cell* 2009, **33**(1):1-13.
248. Price DH: **P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II**. *Mol Cell Biol* 2000, **20**(8):2629-2634.
249. Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q: **Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4**. *Mol Cell* 2005, **19**(4):535-545.
250. Perkins ND: **Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway**. *Oncogene* 2006, **25**(51):6717-6730.
251. Calao M, Burny A, Quivy V, Dekoninck A, Van Lint C: **A pervasive role of histone acetyltransferases and deacetylases in an NF-kappaB-signaling code**. *Trends Biochem Sci* 2008, **33**(7):339-349.
252. Zhong H, SuYang H, Erdjument-Bromage H, Tempst P, Ghosh S: **The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism**. *Cell* 1997, **89**(3):413-424.
253. Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G: **Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1)**. *EMBO J* 2003, **22**(6):1313-1324.
254. Buss H, Dorrie A, Schmitz ML, Frank R, Livingstone M, Resch K, Kracht M: **Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity**. *J Biol Chem* 2004, **279**(48):49571-49574.
255. Mattioli I, Geng H, Sebald A, Hodel M, Bucher C, Kracht M, Schmitz ML: **Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon**. *J Biol Chem* 2006, **281**(10):6175-6183.
256. Schwabe RF, Sakurai H: **IKKbeta phosphorylates p65 at S468 in transactivaton domain 2**. *FASEB J* 2005, **19**(12):1758-1760.
257. Bird TA, Schooley K, Dower SK, Hagen H, Virca GD: **Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit**. *J Biol Chem* 1997, **272**(51):32606-32612.

258. Duran A, Diaz-Meco MT, Moscat J: **Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation**. *EMBO J* 2003, **22**(15):3910-3918.
259. Yeh PY, Yeh KH, Chuang SE, Song YC, Cheng AL: **Suppression of MEK/ERK signaling pathway enhances cisplatin-induced NF-kappaB activation by protein phosphatase 4-mediated NF-kappaB p65 Thr dephosphorylation**. *J Biol Chem* 2004, **279**(25):26143-26148.
260. Zhong H, Voll RE, Ghosh S: **Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300**. *Mol Cell* 1998, **1**(5):661-671.
261. Kiernan R, Bres V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, Jin DY, Emiliani S, Benkirane M: **Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65**. *J Biol Chem* 2003, **278**(4):2758-2766.
262. Chen LF, Williams SA, Mu Y, Nakano H, Duerr JM, Buckbinder L, Greene WC: **NF-kappaB RelA phosphorylation regulates RelA acetylation**. *Mol Cell Biol* 2005, **25**(18):7966-7975.
263. Hoberg JE, Popko AE, Ramsey CS, Mayo MW: **IkappaB kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300**. *Mol Cell Biol* 2006, **26**(2):457-471.
264. Webster GA, Perkins ND: **Transcriptional cross talk between NF-kappaB and p53**. *Mol Cell Biol* 1999, **19**(5):3485-3495.
265. Huang WC, Ju TK, Hung MC, Chen CC: **Phosphorylation of CBP by IKKalpha promotes cell growth by switching the binding preference of CBP from p53 to NF-kappaB**. *Mol Cell* 2007, **26**(1):75-87.
266. Hoffmann A, Baltimore D: **Circuitry of nuclear factor kappaB signaling**. *Immunol Rev* 2006, **210**:171-186.
267. Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD, Berger SL: **Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases**. *Mol Cell* 2001, **8**(6):1243-1254.
268. Polesskaya A, Naguibneva I, Duquet A, Bengal E, Robin P, Harel-Bellan A: **Interaction between acetylated MyoD and the bromodomain of CBP and/or p300**. *Mol Cell Biol* 2001, **21**(16):5312-5320.
269. Li AG, Piluso LG, Cai X, Gadd BJ, Ladurner AG, Liu X: **An acetylation switch in p53 mediates holo-TFIID recruitment**. *Mol Cell* 2007, **28**(3):408-421.
270. Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K: **The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis**. *Proc Natl Acad Sci U S A* 2003, **100**(15):8758-8763.
271. Campbell KJ, Rocha S, Perkins ND: **Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B**. *Mol Cell* 2004, **13**(6):853-865.
272. Dong J, Jimi E, Zhong H, Hayden MS, Ghosh S: **Repression of gene expression by unphosphorylated NF-kappaB p65 through epigenetic mechanisms**. *Genes Dev* 2008, **22**(9):1159-1173.
273. Muddasani P, Norman JC, Ellman M, van Wijnen AJ, Im HJ: **Basic fibroblast growth factor activates the MAPK and NFkappaB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes**. *J Biol Chem* 2007, **282**(43):31409-31421.

274. Tang Y, Zhao W, Chen Y, Zhao Y, Gu W: **Acetylation is indispensable for p53 activation**. *Cell* 2008, **133**(4):612-626.
275. Zeng L, Zhang Q, Gerona-Navarro G, Moshkina N, Zhou MM: **Structural basis of site-specific histone recognition by the bromodomains of human coactivators PCAF and CBP/p300**. *Structure* 2008, **16**(4):643-652.
276. Yang XD, Huang B, Li M, Lamb A, Kelleher NL, Chen LF: **Negative regulation of NF-kappaB action by Set9-mediated lysine methylation of the RelA subunit**. *EMBO J* 2009.
277. Brownell JE, Allis CD: **Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation**. *Curr Opin Genet Dev* 1996, **6**(2):176-184.
278. Jin YH, Kim YJ, Kim DW, Baek KH, Kang BY, Yeo CY, Lee KY: **Sirt2 interacts with 14-3-3 beta/gamma and down-regulates the activity of p53**. *Biochem Biophys Res Commun* 2008, **368**(3):690-695.
279. Hoffmann A, Levchenko A, Scott ML, Baltimore D: **The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation**. *Science* 2002, **298**(5596):1241-1245.
280. Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL: **NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1**. *Cell* 2004, **119**(6):803-814.
281. Krishnakumar R, Gamble MJ, Frizzell KM, Berrocal JG, Kininis M, Kraus WL: **Reciprocal binding of PARP-1 and histone H1 at promoters specifies transcriptional outcomes**. *Science* 2008, **319**(5864):819-821.

CURRICULUM VITAE

Name:	ROTHGIESSER
Vorname:	Karin M.
Geburtsdatum:	11.07.1981
Nationalität:	Deutschland und Peru

Ausbildung:

1988 – 1998	Deutsche Schule "Alexander von Humboldt", Lima - Peru
1999 – 2003	Bachelorstudium in Biologie an der Universidad Peruana Cayetano Heredia, Lima - Peru
2004	Lizentiatabschluss, Titel der Lizentiatarbeit am Instituto de Medicina Tropical Alexander von Humboldt in Lima, Peru: "Elaboration of a diagnostic method for Leishmaniosis in Peru using qPCR"
Seit Oktober 2005	Doktorandin am Institut für Veterinärbiochemie und Molekularbiologie, Universität Zürich

ACKNOWLEDGEMENTS

I would like to start thanking my supervisor, Michael O. Hottiger who gave me the opportunity to do my PhD in his group and supported me throughout my project. I would also like to acknowledge my other two supervisors, Bernhard Dichtl and Andreas Ladurner, for offering helpful suggestions during our yearly meetings.

I am specially grateful to Simona Saccani who patiently helped me with my chromatin IP problems, and to Gioacchino Natoli for his helpful advises and encouragement, and for agreeing to review my thesis. I am also grateful to people from the Functional Genomics Center Zurich (FGCZ) who helped me to establish the custom array, specially Hubert, Cathy and Andrea, without whom this would not have been possible.

I thank all the members of the institute for the technical and moral support given to me during this time, specially Tini for warmly welcoming me to the lab and for introducing me to our project, Moni for helping me with every-day problems, Ingrid for correcting the german version of my summary, Steffi for teaching me how to use the confocal microscope, Ralph for patiently helping me with my computer problems, Taras for the helpful tips about my project, and the rest of the Hottiger group Sandra, Michi, Matthias and Simon for funny summer events and nice atmosphere in the lab. I would also like to thank the girls Sandrine, Tini, Heather, Süheda, Radina, Lucy and Sarah for the nice talks during lunch, the many laughs and their friendship.

A big thank you goes to my family for their support and encouragement during all these years. I am also grateful to my old friends from Peru and my new friends from Zurich who constantly asked me how my cells were growing and whether THE experiment had finally worked. A special thanks goes to my Fito who had to patiently listen to all my good and bad results, thank you for all the comfort and love you give me!

Finally, I would like to acknowledge the Swiss National Foundation, the Krebsliga Zürich and Roche Research Foundation for financial support during this PhD work.